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The Possible Role of the *Staphylococcus epidermidis* LPxTG Surface Protein SesC in Biofilm Formation and its use as a potential vaccine target

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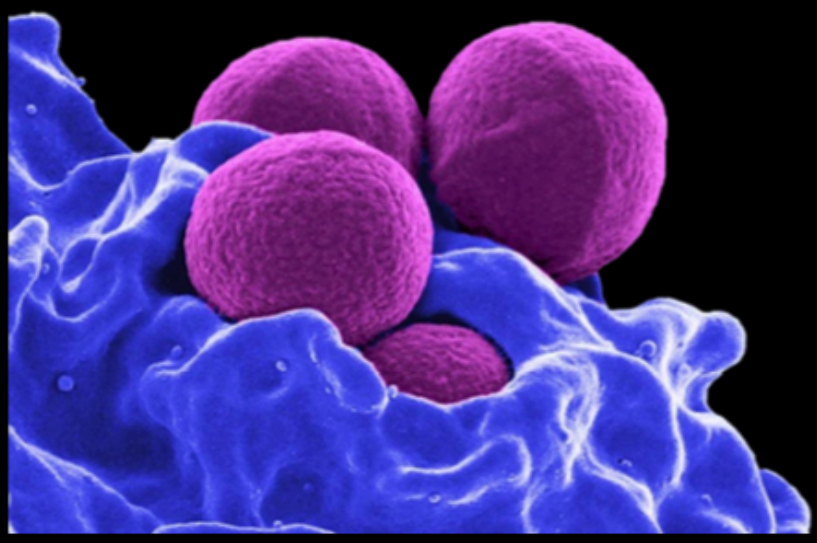

THE POSSIBLE ROLE OF THE STAPHYLOCOCCUSE EPIDERMIDIS LPXTG SURFACE PROTEIN SESC IN BIOFILM FORMATION AND ITS USE AS A POTENTIAL VACCINE TARGET

MAY 2017

KU Leuven
Groep Biomedische Wetenschappen
Faculteit of Geneeskunde
Departement of Klinisch Microbiologie en Immunologie

DE MOGELIJKE ROL VAN HET *STAPHYLOCOCCUS EPIDERMIDIS* LPXTG OPPERVLAKTE-EIWIT SESC IN BIOFILMVORMING EN HET GEBRUIK ERVAN ALS EEN POTENTIEEL VACCIN DOELWIT

Laleh Khodaparast

Promotor:
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Proefschrift voorgedragen tot het behalen van de graad van Doctor in de Biomedische Wetenschappen

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List of abbreviations

aa	Amino Acid
Aap	Accumulation Associated Protein
ADI	Arginine Deiminase
Agr	Accessory Gene Regulator
AI	Autoinducer
Amp	Ampicillin
AIP	Auto-Inducing Peptide
ANOVA	Analysis Of Variance
atlE	Autolysin E Gene
Bap	Biofilm Associated Protein
BHI	Brain Heart Infusion
Bhp	Bap Homologue Protein
bp	Basepairs
BSA	Bovine Serum Albumin
CCD	Charge-Coupled Device
cDNA	Copy Deoxyribonucleic Acid
CFA	Complete Freund's Adjuvant
CFU	Colony Forming Units
ClfA	Clumping Factor A
Cm	Chloramphenicol
Cn	Collagen
CoNS	Coagulase Negative Staphylococci
CRI	Catheter-Related Infection
CRISPR	Clustered regularly interspaced short palindromic repeats
CVC	Central Venous Catheter
CWA	Cell Wall-Anchored
DLL	'Dock, Lock And Latch'
DNA	Deoxyribonucleic Acid
DRI	Device related infections
Ebps	Elastin-Binding Protein Of *Staphylococcus aureus*
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
eDNA	Extracellular DNA
EDTA	Ethylene Diamine Tetra-Acetic Acid ESM

ELISA	Enzyme-Linked Immunosorbent Assay
Embp	Extracellular Matrix Binding Protein
ermC	Ribosomal Methylase-Encoding Gene Of Pe194 For Erythromycin
FBS	Fetal Bovine Serum
Fn	Fibronectin
Glu	Glucose
Gly	Glycine
gmk	Guanylate Monokinase Gene
HeR	Heterogeneously Oxacillin Resistant
hld	Delta-Toxin Gene
HoR	Homogeneous resistant
i.p	Intraperitoneal
i.v	Intravenous
IFA	Incomplete Freund's Adjuvant
IgGs	Immunoglobulin G(s)
JVC	Jugular Vein Catheterized
kDa	Kilo dalton
kHz	Kilohertz
LPxTG	Leu- Pro-X-Thr-Gly; where X means any amino acid
LuxS	S-Ribosylhomocysteine Lyase System
MAs	Monoclonal Antibodies
mec	Methicillin Resistance Determinant
MgrA	a pleiotropic regulator that controls autolysis
MPa	Megapascal
mRNA	Messenger Ribonucleic Acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MSSA	Methicillin-Resistant *Staphylococcus aureus*
NaCl	Sodium chloride
NCBI	National Centre Of Biotechnology Information
OD	Optical Density
OPK	OpsonoPhagocytic Killing
P*blaZ*	Constitutive Lactamase Promoter Module
PBP2a	penicillin binding protein 2a
PBS	Phosphate Buffered Saline
pCN	Plasmid Copy Number
PCR	Polymerase Chain Reaction

PEG	Polyethylene Glycol
PIA	Polysaccharide Intercellular Adhesin
PK	Proteinase K
PMSF	Phenylmethane sulfonyl Fluoride
PNAG	Poly-*N*-Acetylglucosamine
PSM	Phenol-Soluble Modulin
PU	Polyurethane
PVDF	Polyvinylidene fluoride, or polyvinylidene difluoride
QS	Quorum-Sensing
RNA	Ribonucleic Acid
rsbU, V, W	Regulator of the activity of SigB (U, V, W)
rSes	Recombinant Ses
RT-PCR	Reverse Transcriptase PCR
sarA	Staphylococcal Accessory Regulator A
SdrF, G, H	Serine-Aspartate Repeat Protein Family (F, G, H)
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
Ses	*S. epidermidis* Surface (Protein)
SigB	Sigma Factor Sigma B
SigmaB	Alternative Sigma Factor B
SM	Metaperiodate
spp	Species (Plural)
srtA	Sortase A gene
SSP1	Staphylococcal Surface Protein
TcaR	Teicoplanin-Associated Locus Regulator
TCS	Two-Component System
tet	Tetracycline resistance gene
Thr	Threonine
TMB	Tetramethylbenzidine
TSA	Trypticase soy agar or Tryptone soya agar
TTBS	Tris-buffered saline-Tween 20
UV	Ultraviolet
UV/VIS	Ultraviolet/visible
v/v	Volume Volume Ratio
w/v	Weight Volume Ratio
WGA	Wheat Germ Agglutinin
wt	Weight

αSesC	Antibody against SesC

Summary

Staphylococcus epidermidis is the most common cause of device-associated infections among CoNS. It was shown that surface proteins have important roles for attaching and connecting the bacterial cells to the surface of medical devices. Investigation of the most effective antibody against surface proteins in the biofilm formed by *S. epidermidis*, led to the SesC protein as the best candidate.

The purposes of this project were to find the function of this surface protein in biofilm formation by these bacteria and also using the protein as a potential vaccine target.

To explain the role of SesC in the biofilm, first we tried to find a *sesC* natural mutant in *S. epidermidis*. Our investigation suggests that *sesC* is a conserved gene in this bacterial species and specifically present in *S. epidermidis*, not other staphylococci.

Since our efforts to knock out *sesC* were unsuccessful, we used an alternative strategy, which consisted in transforming another staphylococcus, *S. aureus*, with *sesC* and investigating the resulting changes in biofilm formation. We showed that a PIA-dependent strain transformed with and expressing SesC switched to a protein-dependent biofilm formation. This suggests that SesC has an important role in connecting the cells to each other or even to the surface.

Using the antibody against SesC on the biofilm of the transformant strain with *sesC, in vivo* results suggest that SesC can be a good candidate for vaccine development.

Samenvatting

Staphylococcus epidermis is de meest voorkomende oorzaak van biomateriaal-geassocieerde infecties (lijninfecties of katheterinfecties) onder Coagulase-Negatieve Stafylokokken (CoNS). Het is aangetoond dat oppervlakte-eiwitten een belangrijke rol spelen bij het aanhechten van bacteriële cellen aan het oppervlak van medische hulpmiddelen. Uit onderzoek naar het meest effectieve antilichaam tegen oppervlakte-eiwitten in de biofilm gevormd door *S. epidermidis,* kwam het eiwit SesC naar voren als beste kandidaat eiwit.

De doelen van dit project waren enerzijds het achterhalen van de functie van dit eiwit in de biofilmvorming van deze bacteriën en anderzijds onderzoeken of dit eiwit gebruikt kon worden als een potentieel doelwit voor vaccinatie.

Om de rol van SesC in biofilmvorming te achterhalen, probeerden we eerst om een natuurlijk voorkomende mutant van het eiwit te vinden in *S. epidermis*. Ons onderzoek suggereert dat *sesC* een geconserveerd gen is in deze bacteriële soort dat specifiek aanwezig in *S. epidermidis*, maar niet in andere stafylokokken.

Aangezien onze inspanningen om een knockout van het *sesC*-gen te bekomen, niet succesvol waren, gebruikten we een andere strategie die eruit bestond om een andere stafylokokken- soort, *S. aureus*, te transformeren met *sesC* en vervolgens de veranderingen in biofilmvorming te bestuderen. We hebben aangetoond dat een PIA-afhankelijke stam die getransformeerd werd met *sesC* en het eiwit tot expressie bracht, overschakelde naar een eiwitafhankelijke biofilmvorming. Dit suggereert dat SesC een belangrijke rol speelt bij de verbinding van bacteriële cellen met elkaar en met het oppervlak.

De *in vivo* resultaten van het gebruik van het antilichaam tegen SesC tegen de biofilm van de met *sesC*-getransformeerde stam, suggereren dat SesC een goede kandidaat is voor de ontwikkeling van een vaccin.

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CHAPTER I: General Introduction

1.1 Microbiological profile of staphylococci

Staphylococci belong to the *Staphylococcaceae* family, and the genus *Staphylococcus* includes at least 47 species (Becker et al. 2014). These Gram-positive spherical bacteria appear in pairs or grape-like clusters (Figure 1.1) and most species are currently considered as important human opportunistic pathogens (Deighton et al. 1992; Trülzsch et al. 2007).

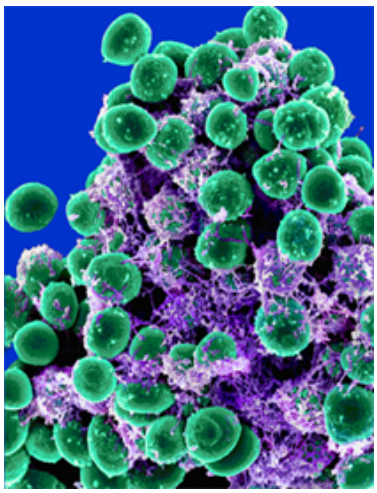

Figure 1.1. Scanning electron microscopy of *Staphylococcus epidermidis* clusters embedded in exopolymeric substance (http://staphylococcusepidermidis.org).

The *Staphylococcus* genus is divided into two large groups based on the ability to produce the enzyme coagulase as observed by the clotting of plasma. The absence of this enzyme is the key characteristic of coagulase-negative staphylococci (CoNS), differentiating them from coagulase-positive staphylococci, mainly *Staphylococcus aureus*. *Staphylococcus epidermidis* is the most frequently isolated member of CoNS (Götz, Bannerman, & Schleifer, 2006).

Other characteristics, such as colony morphology, pigmentation, and hemolysis vary among CoNS. Phenotypic variation is frequently found among these bacteria (Handke et al., 2004), and this may be due to the high level of DNA sequence diversity, even within a single species (Miragaia et al. 2007).

1.2 Pathogenesis of infections by coagulase-negative staphylococci

CoNS have been accepted as the major opportunistic pathogens on the surface of the human body (Becker et al. 2014). Many human infections are associated with CoNS, such as meningitis, pneumonia, enterocolitis, otitis media, urinary tract infections,

surgical wound infections, and a series of medical device-related infections. Medical implants prone to infections include prosthetic heart valves, intravascular catheters, vascular grafts, cardiac pacemakers, intraocular lenses, breast implants, genitourinary prostheses, prosthetic joints, hemodialysis shunts and grafts, central nervous system shunts, and many other surgically inserted appliances (Cervera et al. 2009; Von Eiff et al. 2005; Guggenbichler et al. 2011).

Among all CoNS, *S. epidermidis* has the highest clinical importance. Although *S. epidermidis* had long been considered as a non-pathogenic species, it has gradually been recognized as a relevant opportunistic pathogen for the following reasons:

- 65-90% of all *Staphylococcus* spp. recovered from human infections are *S. epidermidis* (de Allori et al. 2006).
- *S. epidermidis* bloodstream infections are particularly problematic in neonates and it has been recognized as the most common cause of neonatal morbidity and mortality (Cheung & Otto 2010).
- *S. epidermidis* often becomes the major infective agent in compromised patients, such as immuno-compromised patients under immuno-suppressive therapy, AIDS patients and drug abuse patients. In all of these infections, the port of entry into the human body is usually an intravascular catheter (Domingo & Fontanet 2001; Tacconelli et al. 1997).
- Together with *S. aureus*, *S. epidermidis* is the major cause of surgical site infections (Foster 1996).
- *S. epidermidis* ability to establish multilayered, highly structured clusters on artificial surfaces makes it a frequent cause of native valve endocarditis (NVE), and infections related to orthopedic prostheses and vascular catheters (Chu et al. 2008).

1.3 Staphylococci and medical device-related infections

Modern medicine increasingly requires the use of indwelling medical devices to prolong and improve the quality of life for many individuals. However, the frequent use of these devices is associated with side effects as they may become contaminated with bacteria, which adhere to the surface thereby acting as a source of infection. The potential sources of catheter-related infection (CRI) are contiguous skin flora, contamination of the catheter hub and lumen, contamination of infusion fluid, and hematogenous colonization of the intravascular device (IVD) from distant, unrelated

sites of infection (Figure 1.2) (Crnich & Maki 2002; Dunne & Dunne 2002). The majority of infections caused by *S. epidermidis* are acquired in hospitals and are associated with the use of medical devices such as the catheters (Figure 1.2) (Conen et al. 2008). This ability to cause infection is due to adhesion of *S. epidermidis* strains to the device surface which permits multicellular agglomeration with a characteristic three-dimensional structure and physiology which is called "biofilm" (Cherifi et al. 2013).

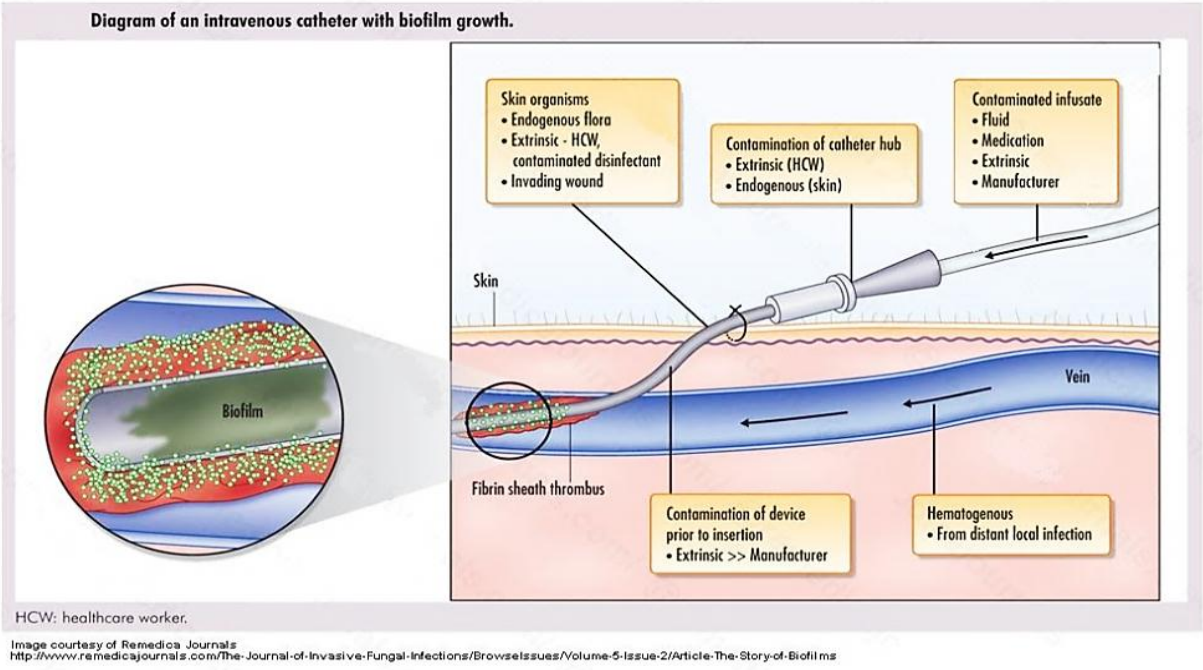

Figure 1.2. Potential sources of bloodstream infections related to the catheter. The contiguous skin flora, contaminated catheter hub and lumen, contaminated infusate, hematogenous colonization of the device from distant infections and contamination of devices prior to insertion are the most common cause of CRI (Crnich & Maki, 2002).

S. epidermidis can cause different infections by spreading and forming biofilms in the body. By attaching to non-biological and biological surfaces and forming biofilms in a self-produced matrix, these bacteria promote their survival and stability in natural environments (Hall-Stoodley et al. 2004). Bacteria on the skin or in the oral cavity can transiently enter the bloodstream and colonize. The irregular detachment of biofilms leads to cycles of bacteremia (Figure 1.3).

Staphylococcal biofilm can additionally be facilitated by the host inflammatory response since host inflammatory molecules promote adhesion to the surface of a device (Hall-Stoodley et al. 2004; Cherifi et al. 2013).

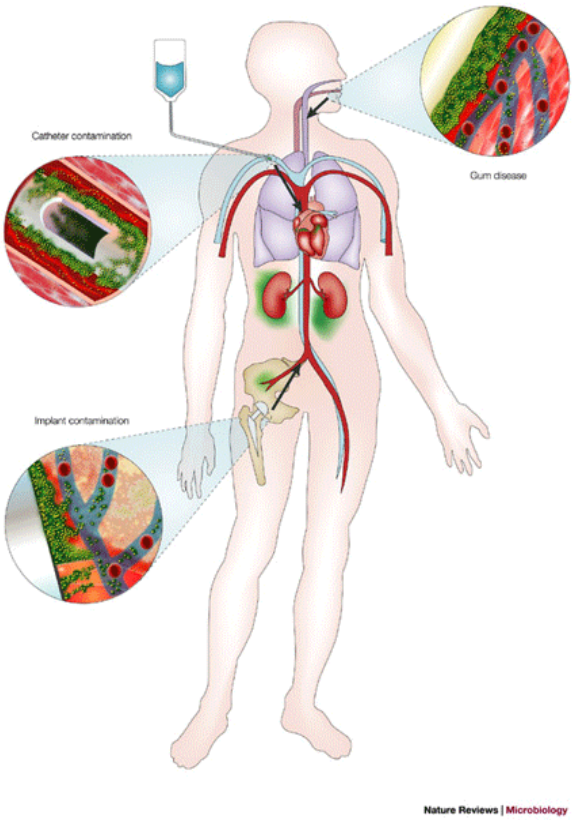

Figure1.3. Schematic showing the examples of possible infectious points of entry of *S. epidermidis* into the body; catheter, hip replacement, and periodontal disease (Hall-Stoodley et al. 2004).

1.4 Development of staphylococcal biofilms

Essentially, biofilms are complex microbial communities in which bacteria get different characteristics from their planktonic counterparts, such as elevated resistance to antibiotics and host defenses. "Therefore, a biofilm can be defined as a community of bacteria that is notoriously resistant to immune system attack and antimicrobial agents" (Høiby et al. 2010).

The complexity of biofilm structure and development, and its importance in both natural and man-made environments, has been recognized and investigated over the past years and this could help to identify the molecular mechanisms that are involved in biofilm development (Hall-Stoodley et al. 2004).

The development of a biofilm occurs in several steps. It starts with the initial adhesion of cells to a surface and their subsequent accumulation, followed by the maturation phase, and finally the detachment of cells or cell clusters from the formed biofilm, which may lead to the dissemination of an infection (Figure 1.4) (Otto 2009).

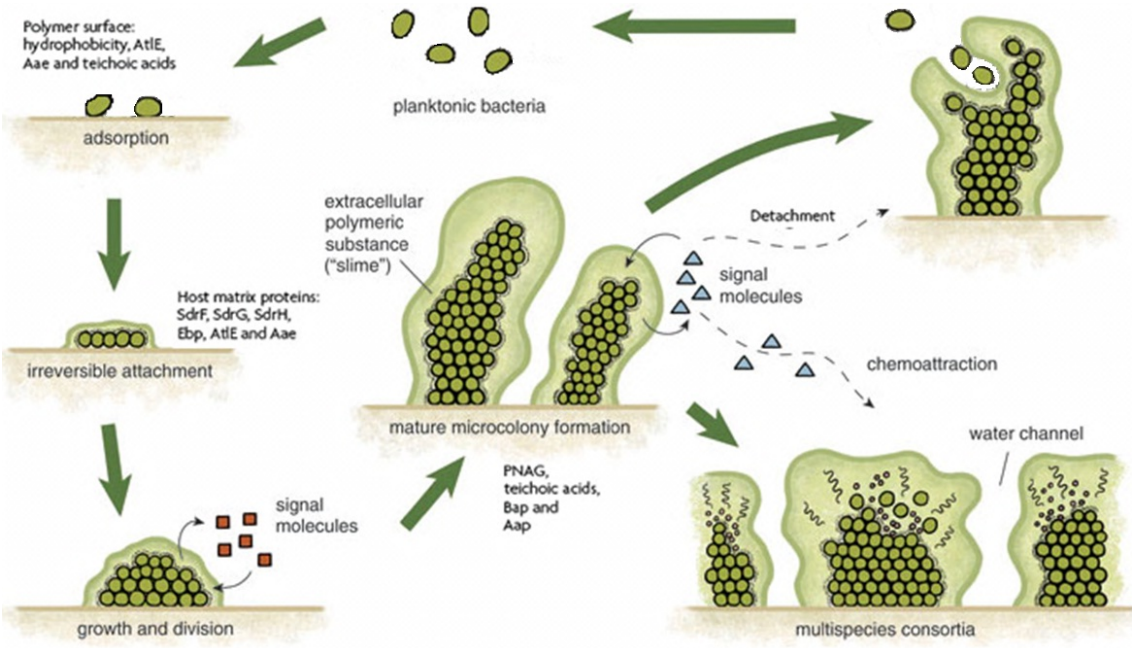

Figure 1.4. Schematic overview of the different stages of staphylococcal biofilm formation. Attachment to uncoated material is mainly related to cell surface hydrophobicity, while dedicated surface proteins mediate adhesion to host matrix-covered devices. Exopolysaccharide, specific proteins, and accessory macromolecules provide intercellular aggregation. Mechanisms of biofilm maturation, detachment, and structuring are poorly understood, but possibly involve quorum-sensing controlled expression of peptides and proteolytic activity in exposed layers of the biofilm. Genome-wide gene expression is significantly different in the biofilm compared to the planktonic mode of growth and includes down-regulation of cell processes (adapted from Otto 2009).

1.4.1 Initial attachment

Direct interaction of a medical device with bacterial cells occurs via non-specific physicochemical forces such as polarity, van der Waal's forces and hydrophobic interactions. Before initial bacterial cell attachment, however, "surface conditioning" often is needed where a surface becomes first coated with adsorbed bridging molecules (Lindsay & von Holy 2006). After insertion, the implanted material rapidly becomes coated with plasma and extracellular matrix proteins such as fibrinogen (Fg), fibronectin (Fn), vitronectin (Vn), thrombospondin and von Willebrand factor (Devine 2003).

Some surface components in bacteria adhere to factors involved in the "conditioning film" and assist to the initial attachment of cells. For example, in *S. epidermidis,* microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) harbor binding sites for human matrix proteins such as fibronectin, collagen, laminin, vitronectin, elastin, and fibrinogen. Staphylococcal MSCRAMMs can attach either

directly or indirectly (Figure 1.5). In *S. epidermidis,* autolysin E (AtlE) is a protein that has an indirect role in initial attachment via release of extracellular DNA (Qin et al. 2007) and a direct role by binding to the extracellular matrix protein Vn and plasma (Hirschhausen et al. 2010; Mack et al. 2013). Mature GehD protein binds to collagens type I, II, and IV (Bowden et al. 2002) SdrF adheres to collagen I via its B domain (Arrecubieta et al. 2009; Mack et al. 2013).

Another important factor is extracellular matrix binding protein (Embp) which has Fn binding activities (Büttner et al. 2015).

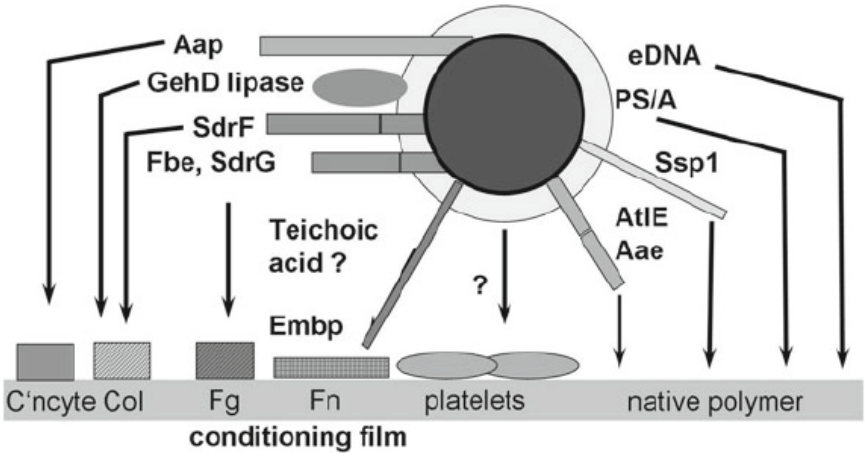

Figure 1.5. Schematic of mechanisms of initial attachment of *S. epidermidis* by different proteins. The surface of medical devices covered by extracellular matrix proteins (ECM proteins) such as fibrinogen (Fg), collagen, fibronectin (Fn) and vitronectin (Vn), thrombospondin after implantation. *S. epidermidis* by different proteins (MSCRAMMs) attach to these ECM proteins. For example, AtlE binds to Vn, SdrF is a collagen binding protein (Mack et al. 2013).

SdrG (Fbe) is a surface-associated fibrinogen binding protein, which binds to host proteins by sharing a similar domain structure in its N-terminal A domain (Sellman et al. 2008).

SesC, a surface protein, can be also involved in the binding of *S. epidermidis* to fibrinogen (Shahrooei et al. 2009).

To initiate attachment, the bacterial cells must be brought in close proximity with the conditioned surface. Transportation of cells to the surface can occur by processes such as liquid flow or Brownian motion (Habimana et al. 2014).

1.4.2 Accumulation phase

After attachment, *S. epidermidis* bacteria first grow two-dimensionally on the device surface, and subsequently accumulate vertically to achieve a multilayer biofilm with three-dimensional structure (Stewart et al. 2015).

After forming an early adherent monolayer, staphylococcal bacteria multiply locally and then reach a mound-shaped cellular aggregation, which is named microcolony. The sessile growth mode of staphylococci is mediated by secreted or surface macromolecules. These macromolecules or extracellular polymeric substance (EPS) (Stewart et al. 2015) may vary between species or even within a strain depending on growth conditions. According to the type of EPS, three accumulation types have been determined for staphylococcal biofilm formation: PIA-dependent/*ica*-mediated, proteinaceous, and extracellular DNA (eDNA)-based biofilm growth.

*1.4.2.1 **ica-mediated biofilm development***

In this mechanism of cell accumulation, production of extracellular polysaccharide intercellular adhesin (PIA) (Figure 1.6a) mediates the cell-cell interaction after the formation of an adherent monolayer. The formation of PIA, also termed polymeric N-acetyl-glucosamine (PNAG), is encoded by the *ica*ADBC operon, the expression of which can be regulated by the regulatory gene product, IcaR (Figure 1.6b). This is the currently best understood biofilm mechanism in staphylococci (Calà et al. 2015).

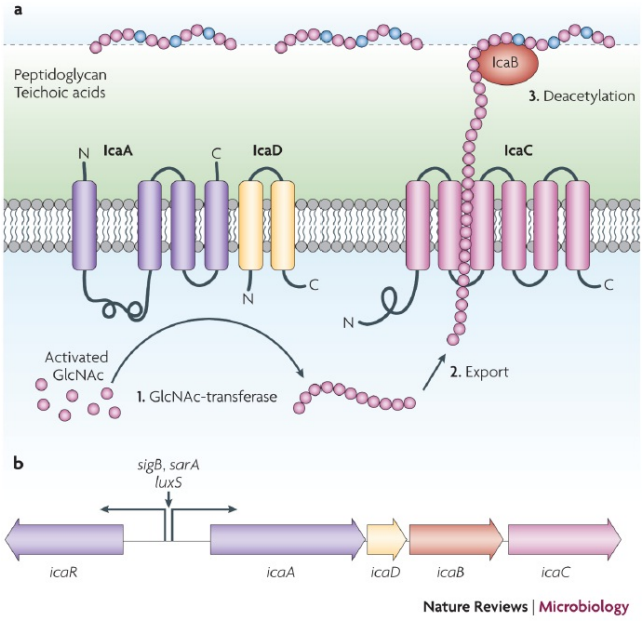

Figure 1.6. The exopolysaccharide PIA/PNAG. The Ica proteins are encoded by the *ica* gene locus, which contains the *icaADBC* operon that generates PIA/PNAG. The *icaR* gene encodes a regulatory protein, IcaR, which regulates the *icaADBC* operon. The exopolysaccharide PNAG/PIA, a partially deacetylated β-1,6-linked *N*-acetylglucosamine homopolymer, is synthesized by the membrane-located *N*-acetylglucosamine transferase IcaA that needs the accessory IcaD membrane protein for its activity. The growing PNAG/PIA chain is exported by the IcaC membrane protein. After exportation, the surface-located IcaB de-acetylase, removes some of the *N*-acetyl groups, giving the polymer a cationic character that is essential for surface attachment (Otto 2009).

IcaA, C and D are membrane-associated proteins, whilst IcaB is mainly found in the extracellular region. IcaA possesses *N*-acetylglucosamine transferase activity, utilizing UDP-*N*-acetylglucosamine as a substrate, and only reaches optimal activity in the presence of IcaD (Figure 1.6a) (Otto 2009).

The presence of IcaAD alone can only produce oligomers of up to a maximum of 20 *N*-acetylglucosamine residues, and IcaC is required for the synthesis of longer chains (Gerke et al. 1998). Furthermore, IcaC is also presumed to translocate PIA to the cell surface. IcaB partially deacetylates PIA, which is a necessary process for attachment of PIA to the cell surface for biofilm formation.

S. epidermidis PIA-dependent biofilms can be liberated/disintegrated by treatment with sodium metaperiodate *in vitro* (Kogan et al. 2006).

1.4.2.2 ***Proteinaceous biofilm accumulation***

PIA, however, seems not to be the only factor for biofilm formation in staphylococci, as biofilm formation has been demonstrated in strains lacking the *ica* genes. Biofilm formation in these strains seems to be mediated exclusively by specific surface proteins such as accumulation-associated protein (Aap) and biofilm-associated protein Bhp (Bap in *S. aureus*). Indeed, in proteinaceous biofilm, surface proteins mediate the cell-cell interaction of adherent monolayers. Aap through repeating G5 domains (five conserved glycine amino acids) contributes to attachment of the cells and also by forming fibrils on the cell surface helps the accumulation phase (Figure 1.7) (Speziale et al. 2014). Some of these surface proteins are characterized by an N-terminal signal peptide and a C-terminal LPxTG motif (Leu- Pro- X-Thr-Gly; where X means any amino acid), followed by a hydrophobic domain and positively-charged tail (Figure 1.8) (Otto 2009; Kogan et al. 2006). Bhp is an LPxTG protein that promotes primary attachment to abiotic surfaces and intercellular adhesion and also helps the

accumulation phase (Calà et al. 2015). In this mechanism, sortase enzyme cleaves the LPxTG motif and bind the N-terminal part of the protein to the cell wall peptidoglycan (Otto 2009; Schneewind & Missiakas 2012).

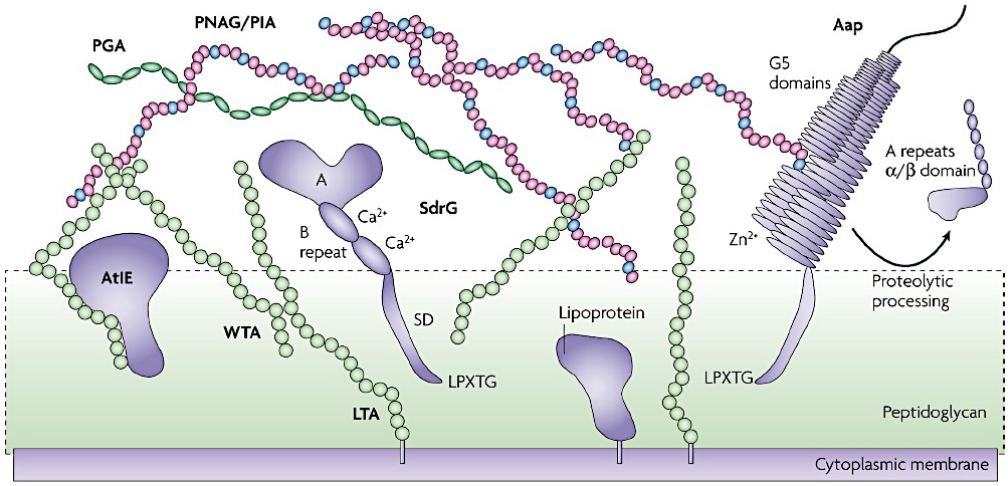

Figure 1.7. Schematic overview of the *S. epidermidis* cell surface. Cell surface proteins harbor a characteristic LPXTG motif at the C-terminus link to the peptidoglycan (Otto 2009).

After cleavage of the precursor by sortase A in *S. epidermidis*, the surface proteins which harbor a characteristic LPxTG motif at the carboxyl group, bind by its threonine and the glycine to an acyl-enzyme intermediate between the active site of sortase A and the carboxyl group of threonine at the C-terminal end of the surface protein. The amino group of cell wall crosses to lipid II by the C-terminal threonine (Figure 1.8) (Bierne & Dramsi 2012).

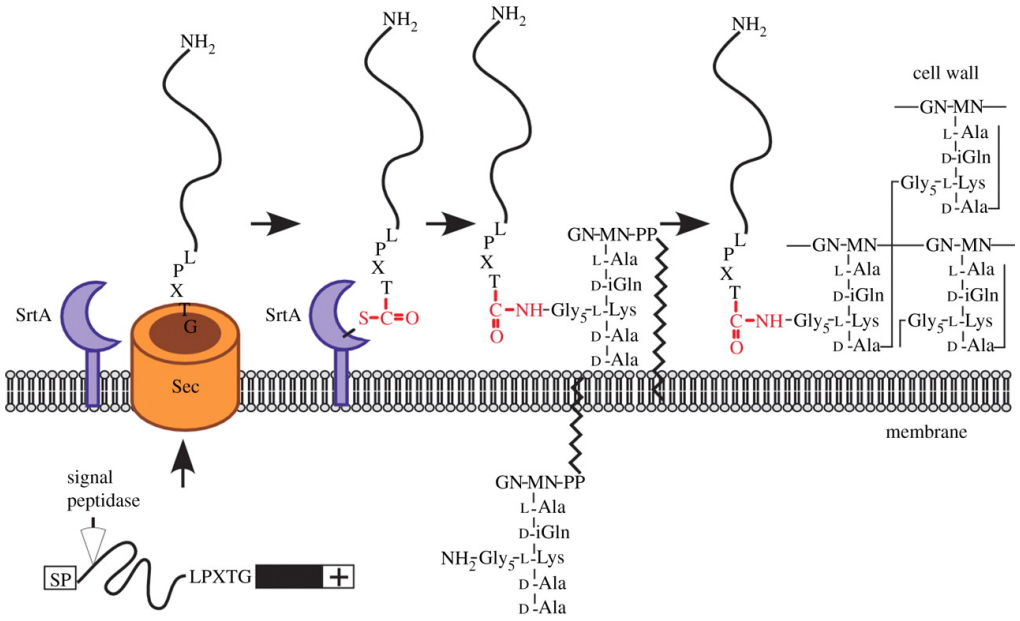

Figure 1.8. Schematic representing the role of sortase A in anchoring LPxTG proteins to the peptidoglycan layer of the Gram-positive bacteria. In *S. epidermidis*, surface proteins are synthesized in the cytoplasm including an LPxTG motif, hydrophobic domain and positively charged tail (Schneewind & Missiakas 2012).

Extracellular matrix binding protein (Embp) is another important protein that contributes in the accumulation phase of proteinaceaus biofilm formation. This protein exists in the cell wall of *S. epidermidis* and helps to protect these bacteria from phagocytosis by macrophages (Christner et al. 2010). Although its exact role is not clear, it is shown that the presence of Embp in *S. epidermidis* is sufficient and necessary for biofilm formation by using defined Embp-positive and -negative *S. epidermidis* (Christner et al. 2010). Proteinaceous biofilms can be decomposed by proteinase K (Fredheim et al. 2009).

1.4.2.3 eDNA biofilm formation

A third adhesive element that allows cells to accumulate in a staphylococcal biofilm is extracellular DNA. eDNA is partially acquired by autolysis of cells within the biofilms. In *Staphylococcus epidermidis* 1457, autolysin E (encoded by *atlE*) activity mediates production of eDNA, and mutants lacking AtlE form significantly less biofilm (Mann et al. 2009; Qin et al. 2007). In several reports, eDNA was shown to be involved in different stages of biofilm formation of some Gram-positive bacteria, including initial adhesion by favorable acid-base interactions, aggregation, biofilm architecture, and mechanical stabilization of biofilms. Whilst the precise mechanism of

eDNA-mediated biofilm formation is unclear (Das et al. 2010; Hu et al. 2012; Peterson & Mei 2013), it is known that DNase I can disperse this kind of biofilm formation.

1.4.3 Biofilm maturation

The maturation phase in staphylococcal biofilm comprises the structural formation of channels and holes between each biofilm cluster which allows the deep cell layers to get the necessary nutrients (Figure 1.4.) (Otto 2013).

The known factors secreted during biofilm maturation are phenol-soluble modulins (PSMs), which contain two-subclasses, α and β. The β subclass has surfactant-like properties that promote the biofilm structuring (Wang et al. 2011). Deletion of a class of PSM peptides (PSMα, PSMβ) significantly affects biofilm mass thickness, roughness, and channel formation, showing that the presence of PSM peptides is important for the development of biofilm (Otto 2013).

PSMs have recently emerged as a novel toxin family defining the virulence potential of highly aggressive staphylococci. They have multiple roles in staphylococcal infections, such as lysis of red and white blood cells, stimulating inflammatory responses and contributing to biofilm development and the dissemination of biofilm-associated infections (Figure 1.9) (Peschel & Otto 2013; Cheung et al. 2014).

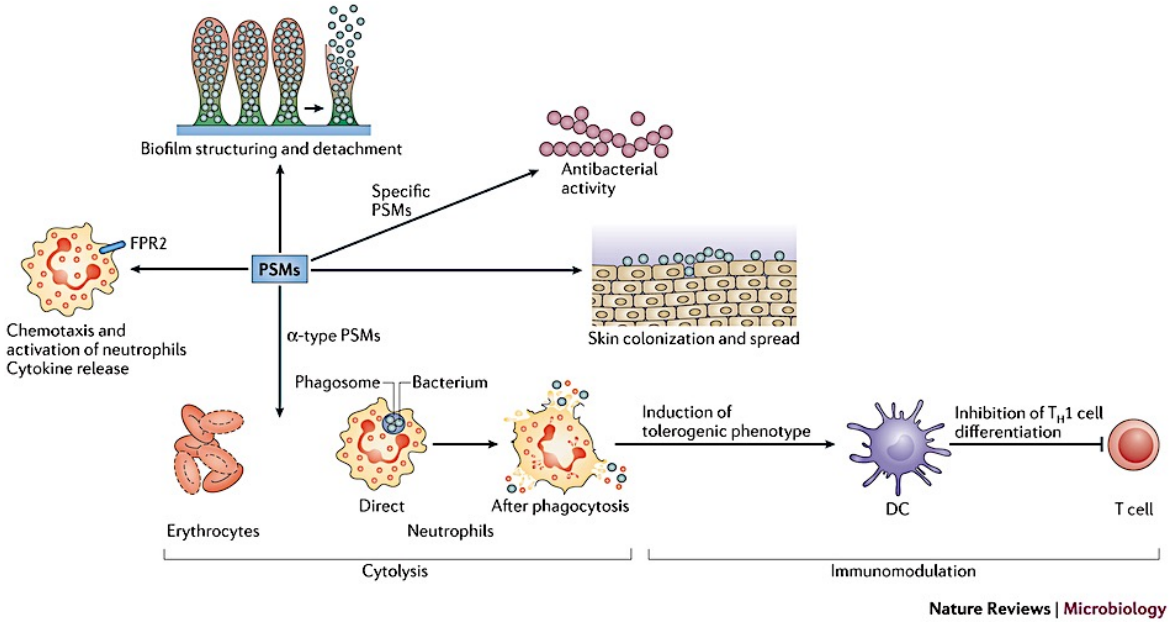

Figure 1.9. Overview of PSM activities. PSMs have different functions, which help in biofilm structuring, detachment, spreading on surfaces, and cytolysis. α-type PSMs have cytolytic activity such as lysis of erythrocytes and neutrophils. PSMs also help to escape from innate host defense by induction of a tolerogenic phenotype in dendritic cells (DCs) and inhibiting T helper 1 cell differentiation (Peschel & Otto 2013).

In *S. epidermidis*, PSMs are secreted with an amino terminal *N*-formyl methionine (fM) which leads to neutrophil activation, chemotaxis and cytokine release (Peschel & Otto 2013). Expression of PSM can also cause biofilm dispersal by the detachment of cells, which is a key mechanism leading to the systemic dissemination of infections involving biofilms (Figure 1.9) (Vuong et al. 2004; Peschel & Otto 2013).

Another factor is Arginine Deiminase (ADI) that functions to boost the biofilm maturation phase by pH homeostasis in *S. epidermidis*. Deletion of the *arc* gene, encoding ADI in *S. epidermidis* 1457 cause the reduction of biofilm by decreasing of the pH, viability, and ammonia synthesis in the biofilm formation (Lindgren et al. 2014).

Bacterial growth in biofilm is different from exponential growth in planktonic mode. Bacterial cells in biofilms shift their physiology towards an anaerobic or microaerobic metabolism. It has also been shown that arginine metabolism is an important factor during this metabolic shift by serving as a sole carbon source (Fey & Olson 2011).

In *S. epidermidis* and *S. aureus*, other polymers such as teichoic acids also help biofilm structuring. They interact via electrostatic interaction with other surface proteins; together they make a complex network in the staphylococcal cell surface (Otto 2013).

Extracellular DNA (eDNA) also helps to order the biofilm structure. The negative charge of DNA may interact with other surface structures and change the type of biofilm (Otto 2013).

Changing pH during biofilm maturation affects the EPS structures. In fact, EPS is unstable at low pH (lower than pH~ 7). Low pH solubilizes the matrix and triggers disassembly of *S. epidermidis* biofilm (Stewart et al. 2015).

The presence of other environmental stresses such as high temperatures (fever), the existence of sub-inhibitory concentrations of antibiotics and osmolarity, affect the quality of biofilm clustering and tend to detach the cells (Boles & Horswill 2011).

1.4.4 Detachment and possible return to the planktonic growth mode

From a mature biofilm, cells may disperse and return to a planktonic mode of growth, thus completing the biofilm life cycle. The detachment of bacterial cells from biofilms is of fundamental importance to the dissemination of infection and to contamination in both clinical and public health settings (Sauer et al. 2002; Stoodley et al. 2001).

It is thought that detached cells express a transitional phenotype between sessile and planktonic states during the first hour after detachment, displaying similar growth kinetics and cell-surface properties as attached biofilm cells (Rollet et al. 2009). Dispersal of cells from biofilms can be caused by external perturbations such as mechanical force or by internal biofilm processes.

The internal factors can be due to the nature of genetic regulation of biofilm such as *agr* quorum sensing system, PSMs and etc. (Wang et al. 2011). The quorum sensing-regulated PSMs, can disrupt non-covalent, electrostatic or hydrophobic interactions and lead to bacterial detachment (Ymele-Leki & Ross 2007).

Environmental factors that can cause biofilm detachment, are: I) factors influencing metabolic activities of bacteria, i.e. starvation of bacteria due to absence of nutrients, limitation of O_2 accessibility (Donlan & Costerton 2002); II) factors causing lysis of biofilm-embedded cells or bacterial death such as exposure to antimicrobial agents; III) factors causing loss of biofilm EPS such as EPS-modifying or -degrading enzymes secreted by the biofilm-embedded bacteria; IV) presence of surfactants produced by CoNS; and V) the presence of shear stress (Hall-Stoodley et al. 2004).

1.5 Genetic regulation of staphylococcal biofilm formation

Regulation of biofilm formation in staphylococci may vary between strains, but the main identified regulators are IcaR, SigB, TcaR, quorum-sensing system, SarA family (such as SarA, SarX, SarZ), ArIR-ArIS two-component system (TCS) and some of the surface proteins. Below, these regulators are briefly described.

1.5.1 Regulation by IcaR

Regulation of the *ica* operon, expression of which results in PIA production (in *ica*- dependent biofilm), is controlled by the DNA-binding protein IcaR that down regulates IcaADBC protein expression (Jefferson et al. 2004). Activation of *icaADBC* can occur through environmental stimuli in an IcaR-dependent manner: addition of sodium chloride or ethanol represses IcaR and consequently enhances *icaADBC* expression. Furthermore, this regulation is controlled by SarA regulator and the global regulatory protein SigB and its activator RsbU, through an unknown pathway (Handke et al. 2007; Jäger et al. 2005) (Figure 1.10a,b).

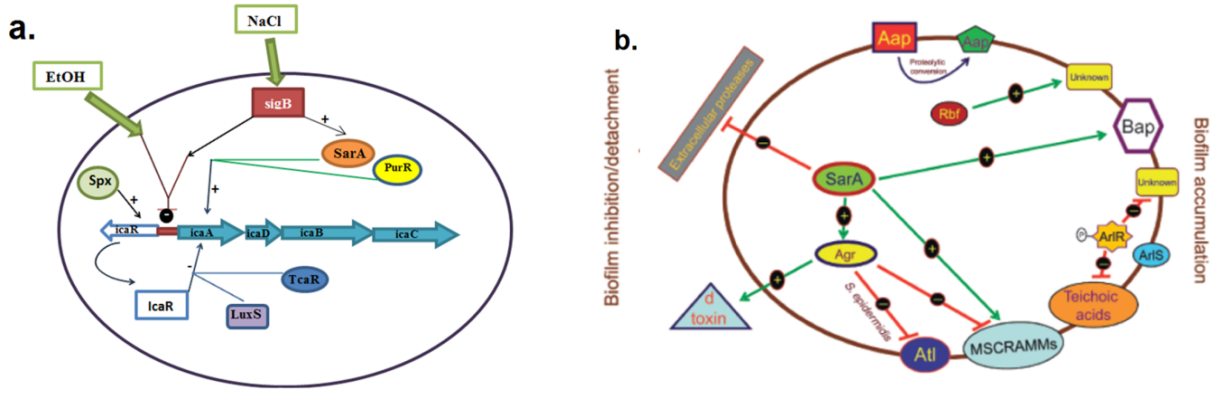

Figure 1.10. a. Genetic regulations of *ica*-dependent biofilm formation; **b.** biofilm mechanisms in staphylococci (O'Gara 2007).

1.5.2 Regulation via SigB

There are currently four identified sigma factors in staphylococci (Pintens et al. 2008); SigA is responsible for transcription of housekeeping genes, SigB is responsible for the transcription of stress response genes, SigS controls expression of genes required for overall fitness and survival, and SigH (Tao et al. 2010) has a demonstrated involvement in competence and prophage integration and excision (Shaw et al. 2008).

SigB is an alternative factor present in different Gram-positive bacteria and *sigB* operon in *S. epidermidis* consists of four open reading frames (ORFs): *rsbU, rsbV, rsbW* and *sigB* (Knobloch et al. 2001).

SigB in *S. epidermidis* and *S. aureus* controls expression of the *ica* operon by repressing transcription of *icaR* and induces biofilm formation under stress conditions such as heat, high osmolarity, high ethanol concentrations, high and low pH as well as oxidizing agents (Pintens et al. 2008; Knobloch et al. 2004). Deletion of *sigB* in *S. epidermidis* reduces biofilm production as repression of *ica* operon by IcaR occurs (Figure 1.10) (Knobloch et al. 2001).

SigB and RsbU regulate the biofilm development of *S. epidermidis* during global stress. Induction of biofilm by NaCl impairs in *S. epidermidis rsbU* mutants, whereas ethanol doesn't affect the activity of this biofilm. Ethanol has a negative impact on *icaR* via SigB and induces biofilm formation (Figure 1.10a) (O'Gara 2007).

1.5.3 Quorum-sensing systems

Quorum sensing (QS) is an intercellular signaling activity responsive to the limitation of nutrients or an increment in bacterial aggregation in the biofilm cluster (Mack et al. 2007). It has been demonstrated that bacteria with QS systems have more activation of virulence and viability linked genes than non-QS bacteria (Olson et al. 2014). Biofilm thickness in *S. epidermidis* is regulated by two QS systems, namely the accessory gene regulator (Agr) and the S-ribosyl-homocysteine lyase (LuxS) system (Olson et al. 2014).

1.4.2.4 The accessory gene regulator quorum-sensing system (agr)

The Agr-QS is identified as an accessory regulator in staphylococcal biofilm. This regulatory system has a central role in the pathogenesis of *S. epidermidis* (Zhang & Ji 2004). The *agr* complex is approximately 3 kDa in size and comprises two primary transcripts, RNAII and RNAIII, which originate from the P2 and P3 promoters, respectively (Figure 1.11) (Kong et al. 2006; Novick & Geisinger 2008).

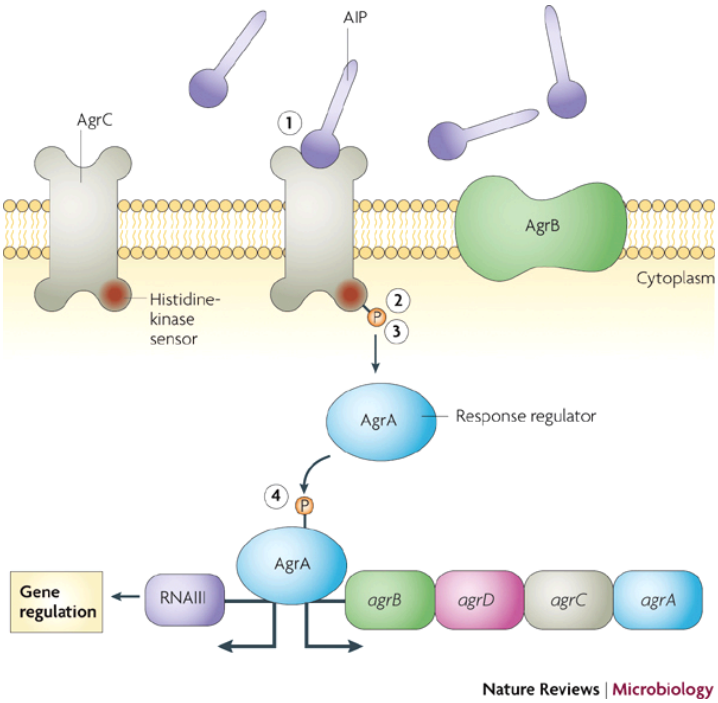

Figure 1.11. The agr gene regulator system in *S. epidermidis*. In *S. epidermidis* as in *S. aureus,* Agr-QS regulates by the production of an autoinducer the P2 promoter and the P3 promoter for transcription of RNAIII. AIPs in *S. epidermidis* induce phosphorylation of the cytoplasmic histidine protein kinase (HPK) domain of the agr signal receptor, AgrC. Then the phosphate transfers to AgrA. AgrA in turn activates transcription from the two *agr* promoters (Cegelski et al. 2008).

The P2-controlled *agr* operon encodes four proteins, AgrA, -B, -C, and -D. AgrD is a membrane protein of which the N-terminal portion is anchored in the membrane by an amphipathic helix that is required for its function and its stability. AgrD has been considered as a pheromone because it signals the state of cell density to all members of the bacterial biofilm (Novick & Geisinger 2008). AgrB is a multipass integral membrane peptidase that is - in cooperation with the signal peptidase SpsB - responsible for exporting and processing AgrD to its active form, namely the auto-inducing peptides (AIP). Within the signal molecule a thiolactone bond between the internal conserved cysteine and the carboxyl terminus is formed giving rise to mature cyclic AIP (Yarwood & Schlievert 2003; Zhang & Ji 2004).

The staphylococcal AIP binds to a transmembrane protein, AgrC. AgrC and AgrA constitute a two-component regulatory system. AgrC is a sensor kinase, which has an N-terminal trans-membrane and a C-terminal domain that is phosphorylated upon ligand binding. AgrA acts as a response regulator with recognition sites in the promoter regions of RNAIII. When the concentration of AIP in the medium is high, a signal transduction cascade is started and, AgrA is activated via phosphorylated AgrC. The DNA binding activity of AgrA will induce transcription from the P2 and P3 *agr* promoters in the late-log phase of growth (Novick & Geisinger 2008; Olson et al. 2014; Koenig et al. 2004) (Figure 1.11).

One of the few toxins produced by *S. epidermidis* is δ-toxin, also called phenol-soluble modulin γ (PSMγ), which is encoded by the *hld* (hemolysin D) gene. This gene is included in the RNAIII transcript that acts as the effector molecule for the *agr* system. Increasing transcription from the P3 promoter, increases the levels of the regulatory RNAIII and concurrently of δ-toxin (Olson et al. 2014).

It is observed that *agr* mutants form thicker biofilms; this increased biofilm thickness is not attributed to cell growth or death, but the inability of cells to detach from the mature biofilm. (Yarwood & Schlievert 2003; Kong et al. 2006).

Deletion of *agr* in the *sigB* mutant compensates the biofilm decrease. Moreover, SigB represses the *agr* system by targeting the P3 promoter and thus the expression of RNAIII (Knobloch et al. 2001).

1.4.2.5 luxS quorum-sensing system

luxS-QS system is present in both Gram-negative and -positive bacteria, but its function in staphylococci is poorly understood. LuxS is an enzyme, which catalyzes the

conversion of S-ribosyl-homocysteine to homocysteine and 4,5-dihydroxy-2, 3-pentanedione (DHPD), which is the precursor of autoinducer-2 (AI-2). In *S. epidermidis,* AI-2 is mainly produced during the logarithmic growth phase, and synthesis is reduced in the stationary phase (Li et al. 2008). Once a threshold level is reached, it acts as a signal which activates the AI-2-specific sensor/regulator system, which in turn directs the expression of various virulence-associated genes (Daniels et al. 2004).

Testing an isogenic *luxS* mutant strain in *S. epidermidis* demonstrated that *luxS* signaling is functional in *S. epidermidis* biofilm formation (Xu et al. 2006). The mutant strain showed an increase of biofilm formation *in vitro* and enhanced virulence in a rat model of biofilm-associated infection compared to the wild type. It was shown that AI-2 regulates the biofilm pathway of *S. epidermidis* by enhancing the transcription of the *ica* operon (Xue et al. 2015).

1.5.4 Regulation by SarA family

SarA is a transcriptional regulator encoded by *sarA* (staphylococcal accessory regulator A) gene (Conlon et al. 2004). The SarA family in *S. epidermidis* consists of different DNA-binding paralogs. Besides SarA, this family includes SarR, SarT, SarU, SarS, SarV, SarZ, SarX, Rot and MgrA (alternatively known as Rat). The transcription of these regulators is strongly dependent on the growth phase (Arciola et al. 2012).

SarA is an essential positive regulator for PIA dependent biofilm (Martı & Penade 2005), but negatively regulates the eDNA biofilm in *S. epidermidis* (Christner et al. 2012).

It is demonstrated that SarA directly regulates *icaADBC* transcription and thus regulates PIA production in a SigB-independent way in *S. epidermidis* (Handke et al. 2007).

SarA regulates the eDNA biofilm by modulating expression of the 1 MDa extracellular matrix binding protein (Embp) and autolysis-dependent release of eDNA (Christner et al. 2012).

Several members of the SarA family are involved in regulation of the *agr* locus, but MgrA is the most important member involved in the activation of the *agr* system as well as in the control by inhibition of the process of cell autolysis (Arciola et al. 2012).

SarX, a SarA family protein, negatively regulates the Agr system. SarX protein binds specifically to the P3 promoter in *S. epidermidis*. However, it is difficult to correlate

SarX-mediated biofilm regulation with altered Agr because both mutation of *sarX* and overexpression of *sarX* activate RNAIII transcription (Rowe et al. 2011).

SarX positively regulates PIA-dependent biofilm. Overexpression of SarX increases *ica* and PIA expression, whereas in *sarX* mutant, *icaA* transcription and PIA expression were decreased and overexpression of SarX did not promote biofilms in an *icaC* mutant (Rowe et al. 2011).

SarZ has also been known as an important regulator in *S. epidermidis* biofilm formation and virulence (Wang et al. 2008). SarZ significantly affects the transcription of the biosynthetic operon for exopolysaccharide biofilm. SarZ also helps the expression of a series of virulence genes, such as genes that influence the expression of lipases and proteases, resistance to the human antimicrobial peptide, and hemolysis (Wang et al. 2008).

1.5.5 Regulation by TcaR

The teicoplanin-associated locus regulator (TcaR) is a member of the multiple antibiotic resistance regulator (MarR) family that exists in both *S. epidermidis* and *S. aureus*. It has been shown that this factor is involved in teicoplanin and methicillin resistance. By Northern blot analysis, it has been shown that inactivation of *tcaR* increases transcription of *icaADBC* in *S. aureus* and in *S. epidermidis*. Thus, TcaR negatively regulates *ica* operon expression (Jefferson et al. 2004; O'Gara 2007).

In 2010, Chang et al. reported that six dimers of TcaR are capable of binding three DNA segments (33 bp) close to the IcaR DNA-binding region with different affinities and their repressor activity is directly interrupted by salicylate and different classes of natural antimicrobial agents (Chang et al. 2010). However, deletion of *tcaR* did not have any effect on the rate of PIA production and on the binding of bacteria to the polystyrene, while deletion of *icaR* leads to an enhancement in both attachment and PIA biosynthesis. Furthermore, deletion of both *icaR* and *tcaR* increases the rate of PIA production 100-fold more than in an *icaR* mutant and 500-fold more than the wild type. It was concluded that TcaR is a weak repressor of *ica* operon transcription while IcaR is a strong one (Jefferson et al., 2004).

1.5.6 Functions of the surface proteins in biofilm formation and infection by *S. epidermidis*

The staphylococcal surface is decorated with cell wall-anchored (CWA) proteins which are covalently bound to the peptidoglycan layer (Foster et al. 2014). In 2005, Bowden *et*

al. characterized eleven genes encoding putative CWA proteins for *S. epidermidis*. The CWA proteins include the MSCRAMMs and the G5-E repeat family, which play both an important role in biofilm formation (Figure 1.12).

So far, the functions of several surface proteins Aap, Bhp, AtlE, SdrF and SdrG were described.

One of the MSCRAMMs, serine–aspartate repeat-containing protein G (SdrG) from *S. epidermidis* has been identified as a fibrinogen-binding protein. The N-terminal A region of SdrG contains three subdomains with IgG-folded domains (Bowden et al. 2008). This protein binds fibrinogen using the 'dock, lock and latch' (DLL) mechanism. This binding mechanism involves the docking of the ligand in between two SdrG subdomains (N2 and N3) by the movement of a C-terminal extension of one subdomain covering the ligand and insertion of a complementary beta-sheet in a neighboring G′ strand in the N3 subdomain (Bowden et al. 2008; Foster et al. 2014).

SdrF, another MSCRAMM, mediates binding to collagen (type I). SdrF, like SdrG, has a cell-sorting LPxTG motif, a region that spans the *S. epidermidis* membrane and a C-terminal cluster of positively charged residues (Arrecubieta et al. 2007). This protein contains additional B-repeats that by using the DLL mechanism bind to the collagen IV. This protein also has the N-terminal A region that contains three folded subdomains, N1, N2 and N3 (Foster et al. 2014).

Aap in *S. epidermidis* and surface protein G (SasG) in *S. aureus* are proteins that form fibrils to promote biofilm formation; the N-terminal of these proteins link to the wall and span via Zn^{2+}-dependent G5 domains (Figure 1.12) (Foster et al. 2014; Otto 2009).

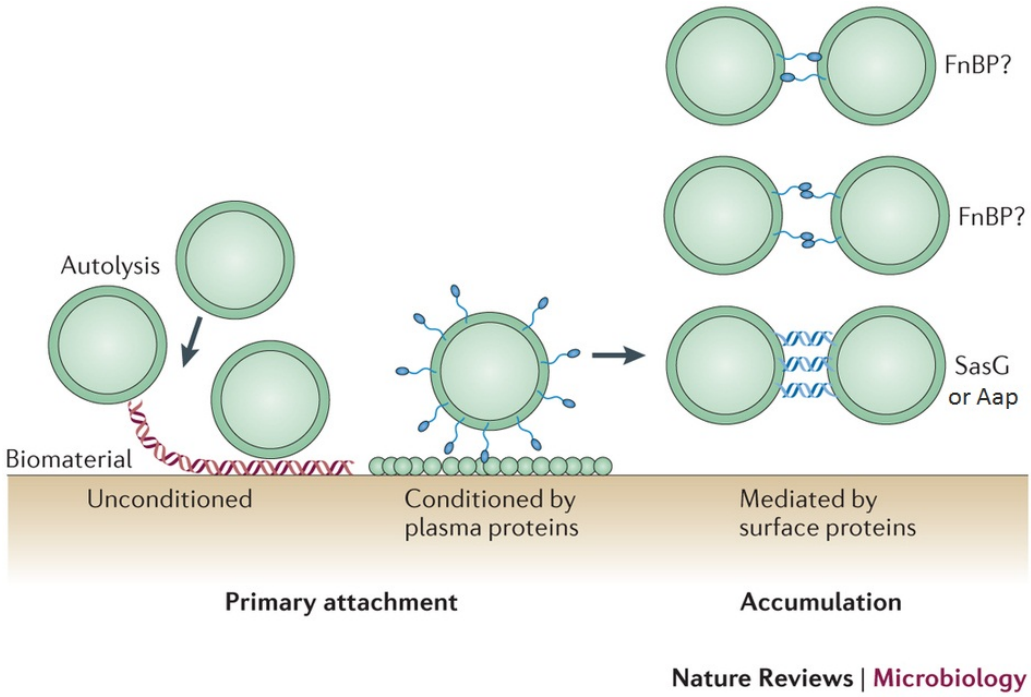

Figure 1.12. Roles of CWA proteins in biofilm formation. Cell wall-anchored (CWA) proteins promote attachment to the surfaces that have been conditioned with host plasma proteins. Extracellular DNA that is released by autolysins can promote attachment to unconditioned surfaces. When the A domain of Aap of *S. epidermidis* or *S. aureus* surface protein G (SasG) is removed, the G5–E repeats promote cell–cell aggregation and biofilm accumulation. Fibronectin-binding proteins (FnBPs) and other surface proteins promote accumulation either by protein–protein interactions.(Foster et al. 2014).

Furthermore, analysis of primary sequences of *S. epidermidis* demonstrated 7 other hypothetical surface proteins (SesA, SesB, SesC, SesE, SesG, SesH and SesI) of which the structural formation is very similar to the previously described cell wall-anchored proteins of other Gram-positive cocci, especially *S. aureus* (Bowden et al. 2005). However none of the Ses proteins are direct homologues of the *S. aureus* proteins. Secondary and tertiary structure predictions suggest that most of the Ses proteins are composed of several contiguous subdomains, and the majority of these predicted subdomains are folded into β-rich structures.

The function of these proteins is not clear yet, but it was shown that *sesB*, *sesC* and *sesE* are present in all *S. epidermidis* strains examined (Bowden et al. 2005; Thomas et al. 2014). For *sesA,* it was also reported that it was present in all *S. epidermidis* strains (Bowden et al. 2005), but some researchers reported strains without *sesA* (Guss et al. 2009).

The *sesI* gene is not present in the normal *S. epidermidis* flora of healthy man, but is present in approximately 50 % of clinical isolates causing invasive infections (Söderquist et al. 2009).

Surface proteins in different strains have important roles in biofilm formation; Shahrooei et al. tried to determine the roles of these proteins in *S. epidermidis* (Shahrooei et al. 2012). They investigated the expression of these genes during different time points of biofilm formation in sessile bacteria compared to their planktonic counterparts in *S. epidermidis* (Shahrooei, 2010). For this study, DNA and RNA were extracted from biofilm attached to the seven mm catheter fragments (Arrow International, Reading, Pa.) in different time points (0, 10, 35, 60, 120, and 180 min). Gene expression was quantified by cDNA/gDNA ratio for each gene and the significant differences were quantified ($p<0.05$; 1-way-ANOVA) at any given time point compared with the time point zero (Table 1.1).

Table 1.1. Gene expression pattern *in vitro* of *ses* genes in sessile bacteria compared to their planktonic counterparts. **↑:** Upregulation, **↔:** No changes (Shahrooei, 2010)

ses gene	0 min	10 min	35 min	60 min	120 min	180 min
sesN	↔	↑	↑	↑	↑	↑
sdrG	↔	↑	↑	↑	↑	↑
sesO	↔	↑	↔	↔	↔	↔
sesE	↔	↑	↔	↔	↔	↑
sesL	↔	↑	↑	↑	↑	↑
sesJ	↔	↑	↑	↔	↔	↑
sesK	↔	↑	↑	↑	↑	↑
sesP	↔	↔	↑	↔	↔	↑
sesM	↔	↔	↔	↔	↔	↔
sesB	↔	↑	↑	↑	↔	↑
sesQ	↔	↑	↑	↑	↑	↑
sesC	↔	↔	↔	↔	↔	↔
sdrF	↔	↔	↔	↔	↔	↑

Subsequently, Shahrooei (2010) purified polyclonal antibodies against rSes proteins, and the effect of these antibodies on the rate of biofilm formation of *S. epidermidis* was evaluated. An antibody against SesC protein could significantly reduce biofilm formation by *S. epidermidis* 10b (Figure 1.13).

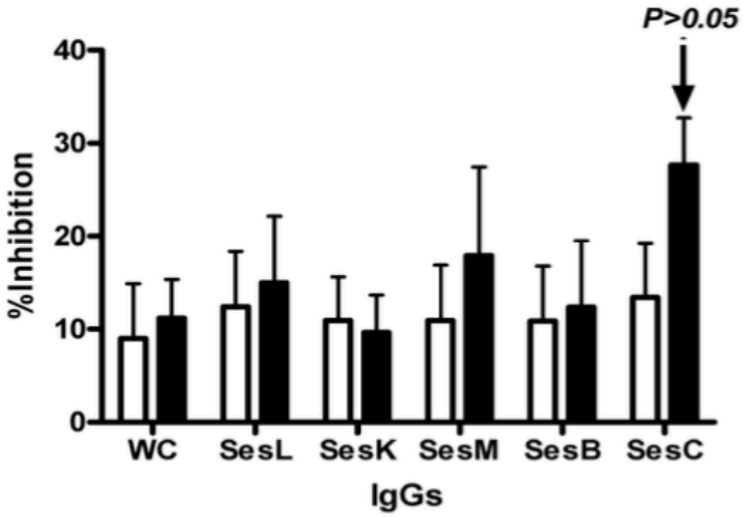

Figure 1.13. Inhibition of primary attachment of *S. epidermidis* 10b cells to polystyrene in presence of total IgGs purified from pre-immune (□), and anti-whole cell (WC) or anti-SesL, K, M, B, C sera (■) (Shahrooei, 2010)**.** Bacteria were mixed with IgGs and incubated for 2 h at 4°C and then pipetted into the wells. After 2 h incubation at 37°C, plates were washed and stained with crystal violet and the OD_{595} was measured. The error bars indicate standard deviations. Data are the average of 8 measurements in 2 independent experiments.

They found also that SesC is a MSCRAMM with the ability to bind to fibrinogen (Shahrooei et al. 2009).

SesC encodes a 676-amino acid (aa) protein with a predicted molecular mass of 75 kDa. The cytoplasmic precursor of SesC contains a 35-aa N-terminal signal peptide (predicted using the SignalP server at http://www.cbs.dtu.dk /services/SignalP/), a 37-aa C-terminal LPxTG sorting signal, and a large extracellular domain. The N-terminal signal is required for Sec-dependent secretion and is cleaved by signal peptidase. The C-terminal signal is needed for cleavage between the threonine and the glycine of the LPxTG motif and for attachment to peptidoglycan by sortase (Shahrooei et al. 2009).

The understanding of the different mechanisms contributing to biofilm formation can help to develop a vaccine against infection by these bacteria and, since the function of SesC is not clear yet, in this study we tried to explain the function of this protein.

CHAPTER II: Aims of this study

Aims of this study

For the last few years, bacterial biofilm formation has been considered as one of the more important infectious causes of irreparable damage to the environment and health. *S. epidermidis* is one of the prime organisms responsible for damage due to biofilm.

Staphylococcal biofilm formation has a complex molecular mechanism, which has only been partly clarified. The roles of some surface proteins in this complex process have been determined.

To date there remain surface proteins in *S. epidermidis* of which the function is not fully understood. Understanding their function could help to find novel strategies to combat biofilm formation.

It was reported that a polyclonal antibody against SesC protein can significantly reduce biofilm formation by *S. epidermidis*. Following this finding, in the present study we had the following aims:

i. To investigate the function of SesC in the biofilm formation of *S. epidermidis*
ii. To establish a new *in vivo* model to further evaluate the potential use of SesC as a target for vaccine development and its role in device-related infection (DRI) *in vivo*
iii. To evaluate SesC or other Ses proteins to be used as a potential vaccine target

Taken together, understanding the role of SesC in biofilm formation may help to find a way to comprehend *S. epidermidis* biofilm and by conjugating the best epitopes of different proteins we may reach the best antigen to immunize humans against this bacterium.

CHAPTER III: Construction of *sesC* mutants

3.1 Introduction

Introduction of mutations is a valuable method to understand the function of proteins (Loeb 1989). There are several methods to generate random mutations in a specific protein, in addition to a knockout of a target gene or knockdown of mRNA by using the Antisense-RNA method (Wagner & Simons 1994). To explain the role of SesC, we tried to construct *S. epidermidis ses*C- mutants.

3.2 Materials and Methods

3.2.1 Knockout of *sesC*

Conventional mutant strategy was our first attempt to knock out *sesC*, which started by designing and making the relevant cassette gene.

In this study, a temperature-sensitive *E. coli/Staphylococcus* shuttle vector pBT2 (Brückner 1997), was used. First, primers for PCR were designed (Table 3.1) according to the genomic sequence of *S. epidermidis* ATCC12228. Genome regions flanking the *sesC* gene were PCR amplified and ligated into the pGEM-T Easy vector (Promega, Madison, WI). Subsequently, the PCR fragments were cloned into pBluescript-II SK vector (Stratagene) resulting finally in pBS-*sesC*-up and pBS-*sesC*-up/*sesC*-down, respectively. The *tetM* gene rendering the host cell tetracycline resistant was PCR-amplified using pCN36 (Charpentier et al., 2004) as template DNA, then *Xho*I/*Apa*I-digested and cloned between *sesC-up* and *sesC*-down in pBS. The *Bam*HI/*Kpn*I-digested knockout cassette *sesCup-tetM-sesCdown* was next inserted into pBT2, yielding pBT2-*sesCup-tetM-sesCdown.*

Competent cells for electroporation were prepared by washing exponentially growing cells with 10% glycerol. This plasmid was electroporated into different *S. epidermidis* strains. Electroporation was performed at room temperature in a 0.2 cm cuvette (BTX Electroporation Cuvettes PlusTM), at a setting of 2.5 kV, 25 μF, 100 ohm for 2.5 ms using a gene pulser (BTX Electroporator ECM 630).

3.2.2 Knock-down of SesC using Antisense RNA

Antisense RNAs act by shifting their target gene transcription through interference or transcription attenuation in bacteria (Thomason & Storz 2010). Some of the antisense RNAs can act like trans-encoded sRNAs to block ribosome binding and translation. To construct an antisense-RNA, a 372-bp part of *sesC* was amplified (Table 3.1) and cloned into the pLI50 vector (Table 3.2) using restriction site *Kpn*I and *Bam*HI, ligated at the 3' end of plasmid pLI50 and electroporated into *S. epidermidis* strains

st1 (a clinical isolate), TU3298 (Tu), 10b, ATCC12228 and RP62A. The presences of plasmid in the transformant strains were confirmed by plasmid extraction.

TABLE 3.1. Primers used in this study.

Primer	Sequence (5'-3')
sesC-SF	GTTGATAACCGTCAACAAGG
sesC-SR	CATGTTGATCTTTTGAATCCC
sesC F1 -LL	TGCCAAGCAAATTGATGGTCTTGCA
sesC F2 -LL	ACTCTGCGATTGAAGGCAAGGCA
sesC R1 -LL	GCTTGTTGGATTTTGTCAGCGATGG
tetM pCN36 F	GTTTAACTCGAGACCTAGGCAAATATGCTCTTACGTGC
tetM pCN36 R	CTATGAGGGCCCGCCGCGGAAATATTGAAGGCTAGTCAG
Up F LL: BamHI	ATGGATCCGGTAAACATAAATAGTCCTCCTTCG
Up R LL: XhoI	ATCTCGAGCACATACTCCTTTAATAAAGTTACCT
Down F LL: ApaI	ATGGGCCCAAGCCAAAACTTGTCGAATTCTC
Down R LL:KpnI	ATGGTACCTCCACAAAATAATAGCACAAAGATG
sesC- antis-F KpnI	ATGGTACCAGTACGACAACAAATCAATCCG
sesC-antis-R BamHI	ATGGATCCTCTTTGTCGGTCAATACTTTGC

TABLE 3.2. Plasmids used in this study

plasmids	Characteristic(s)	Source or reference
pGEM®-T Easy	Cloning Vector	Stratagene
pBluescript II SK	Cloning Vector	Promega
pCN36	*E. coli-Staphylococcus* shuttle vector, Apr-Tet	(Charpentier et al. 2004)
pBT2	*E. coli-Staphylococcus* shuttle vector Apr-Erm	(Brückner 1997)
pLI50	*E. coli-Staphylococcus* shuttle vector- Apr-Cm	(Lee et al. 1991)
pCN68	*E. coli-Staphylococcus* shuttle vector- Apr-Erm	(Charpentier et al. 2004)

3.2.2.1 Detection of SesC knockdown

Western blot analysis of SesC in *S. epidermidis* clones transformed by antisense-RNA was performed as described (Nakashima & Miyazaki 2014). Briefly, bacterial cells of 10 overnight cultures grown at 37°C in BHI, were harvested by centrifugation, re-suspended within lysis buffer [10 mM Tris/HCl, 10 mM EDTA, 1 mM phenyl-methane sulfonyl fluoride (PMSF, pH 7.5), 100 µg lysozyme/, 100 µg lysostaphin/] and incubated for 1 h at 37°C. Then, cells were broken by passing through a French press at 69 MPa (SLM Aminco) three times and followed by sonication (Bath sonicator-Branson 2510, 42 kHz) of samples on ice. Thereafter, soluble proteins were

separated from the insoluble proteins by centrifugation (13000 rpm 4°C, 5 min). The proteins (25 mg) were separated using 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto PVDF membranes. The primary antibody (1:5000 dilution of a rabbit anti-SesC polyclonal antibody in TTBS) was added to the membranes and incubated overnight in 4°C. After three washes with TTBS, secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:2500 in TTBS+1% skim milk)) was added and incubated for 2 h. Finally, after washing three times, the SesC proteins were detected by ECL western blotting detector (Amersham^{TM}, GE Healthcare) in combination with a CCD camera to visualize the presence of SesC.

3.3 Results and Discussion

The correct cassette pBT2-*sesC*up-*tetM*-*sesC*down was made in *E. coli* DH5α, however, transformation in *S. epidermidis* was not successful and we have not succeeded in knocking out of *sesC* using this strategy.

The second strategy, antisense RNA, also didn't work. Western blots showed no changes in expression of SesC in transformant *S. epidermidis* strains in comparison with their parental strains (Figure 3.1).

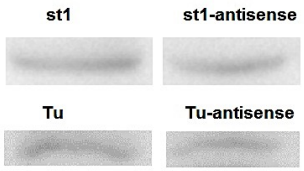

Figure 3.1. Immunodetection of SesC in *S. epidermidis* strains st1 (a clinical isolate) and TU3298 (Tu), and their respective transformants containing pLI50-antisense *sesC*.

Uptake of foreign DNA by *S. epidermidis* is not easy, possibly because of the presence of a CRISPR/Cas system in these bacteria (Figure 3.2). CRISPR loci produce CRISPR RNA which after transcription, hybridizes to the foreign DNA sequences and cleaves it (Otto 2012). Another explanation is that the *sesC* gene may be an essential gene for this strain.

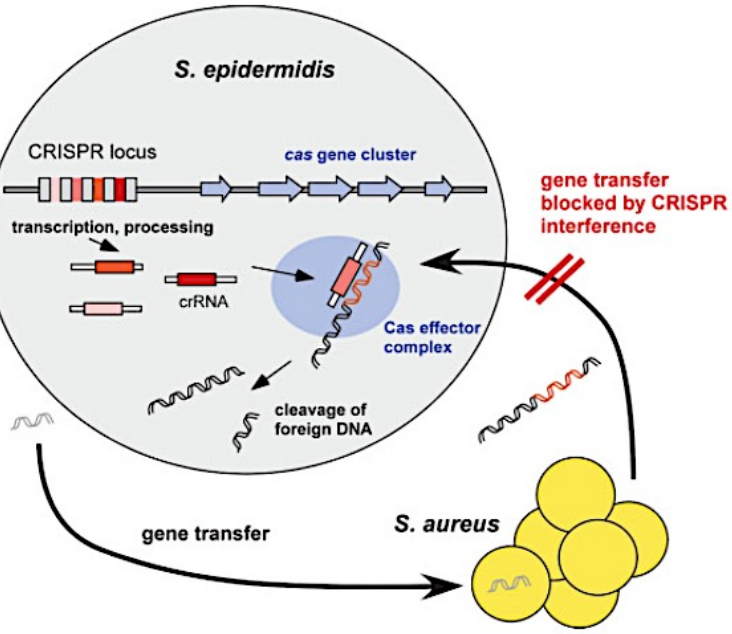

Figure 3.2. Transferring genes between *S. epidermidis* and *S. aureus* are subject to CRISPR/Cas interference. *S. aureus* produces a large variety of toxins from mobile genetic elements in opposition to *S. epidermidis*, which lacks these genes. The CRISPR/Cas system in *S. epidermidis* destabilizes the foreign DNA (Otto 2012) .

CHAPTER IV: *SesC* as a genetic marker for identification of *Staphylococcus epidermidis*

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Research paper

sesC as a genetic marker for easy identification of *Staphylococcus epidermidis* from other isolates

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ABSTRACT

Staphylococcus epidermidis is one of the major concerns with respect to hospital-acquired infections. Therefore, a rapid and easy method to identify at species level *S. epidermidis* isolates out of a broad range of bacteria is necessary.

Based on earlier studies, the *sesC* gene encoding a *S. epidermidis* surface protein revealed to be a highly conserved gene in this species. By means of an easy and inexpensive PCR assay, the presence of *sesC* was checked in 438 clinical staphylococcal isolates. Results showed that *sesC* is specifically present in all *S. epidermidis*. In conclusion, the *sesC* gene can be exploited as a genetic marker in order to distinguish *S. epidermidis* from other isolates.

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4.1 Introduction

S. epidermidis being a prominent bacterial species of the normal human skin microbiota is nowadays recognized as an important opportunistic pathogen, which can easily attach to medical devices and consequently cause severe infections. Globally, *S. epidermidis* represents the most frequent (about 75% to 90%) species among the coagulase-negative staphylococci (CoNS) isolated from clinical samples and is the most common source of infections on indwelling medical devices (Piette et al. 2009; Sarathbabu 2013).

Since identification of these bacteria based on morphology and biochemistry is laborious, and regular diagnosis of a bacterial infection can take some days or even longer after sampling, a rapid and reliable technique to identify this bacterium is most necessary.

In recent years, a most commonly used method to identify and screen bacterial isolates is PCR amplification of a species-specific marker gene (Järvinen et al. 2009). PCR is a simple method which can be carried out immediately on a sample without prior DNA purification, using a single colony (Sheu et al. 2000). The marker gene envisaged is a species-specific, conserved gene of which the presence is most probably essential within that species. If such gene is found, the conserved sequence can be exploited to identify the matching bacterial species (Zeigler 2003).

In this study, we investigated if *sesC* is such a gene, which specifically marks *S. epidermidis* strains or isolates and can be used for the identification of these bacteria. *sesC* encodes *Staphylococcus epidermidis* surface protein C (SesC) which consists of 676 amino acids including a sortase-recognized LPxTG motif (Bowden et al. 2005). It has previously been demonstrated that the SesC protein is more strongly expressed in *S. epidermidis* biofilm-associated than in planktonic cells.

As *S. epidermidis* causes frequently biofilm-related infections for which culturing is difficult, this strengthened the choice of *sesC* as a candidate marker gene. This idea was further supported by the fact that *sesC* could not be deleted in an attempt to get insight in its function, suggesting it might be essential for the viability of the species. In addition, *in silico* genome analyses did not reveal homologous genes in other staphylococci. All these findings suggested that *sesC* could be used as a genetic marker for *S. epidermidis*.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth media

A total of 438 clinical staphylococcal isolates derived from different infection or bacteremia sources from different patients, were collected from the University Hospital Leuven – Gasthuisberg and first identified by mass spectrometry (MALDI TOF, Bruker) which is an accurate genotypic identification system (99.3% of strains correctly identified) (Loonen et al. 2012), then went through the VITEK® 2 identification system (BioMérieux) (100% of strains correctly identified when both methods are performed simultaneously) (Loonen et al. 2012). All *Staphylococcus* spp. were grown in Brain Heart Infusion medium (BHI, Oxoid) or Tryptone Soya Agar (TSA, Oxoid).

4.2.2 Primers

All primers used in this work, are represented in Table 4.1. They were designed with Primer Express 2.0 software (Applied Biosystems, USA) and purchased from Eurogentec (Seraing, Belgium). *sesC*-related primers were all based on the genomic DNA sequence of *S. epidermidis* ATCC 12228 as available in the National Center for Biotechnology Information (NCBI) databank. (Gene ID of *sesC* (SE2232): 1056520; protein accession no. NP_765787.1).

Table 4.1 Primers used in this study.

Primer	Sequence (5'-3')
sesC-F1	GTTGATAACCGTCAACAAGG
sesC-F2	TGCCAAGCAAATTGATGGTCTTGCA
sesC-F3	ACTCTGCGATTGAAGGCAAGGCA
sesC-R1	CATGTTGATCTTTTGAATCCC
sesC-R2	GCTTGTTGGATTTTGTCAGCGATGG

4.2.3 Colony PCR

In order to perform colony PCR, bacterial isolates were grown overnight on TSA plates, a single colony was picked by sterile pipet tip (without touching the TSA agar) and cells were suspended in 20 µl of sterile distilled water. Then, 4 µl of this bacterial suspension was added as DNA template to a PCR tube containing 5 µl of 2x PCR Master Mix (Promega) and 0.5 µl of sesC-F1 and sesC-R1 primers rendering the final sample volume of 10 µl. The following PCR conditions were applied to amplify the *sesC* region using the GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems): 94°C for 5 min, followed by 30 cycles of 30 seconds at 94°C, 30 sec at 55°C, and 1 min at 72°C, and at last a final extension step at 72°C for 7 min. Appropriate positive and negative controls were included to compare the efficiency of amplification and to prove the absence of significant PCR inhibition.
In order to determine the sensitivity of the colony PCR assay, 2-fold serial dilutions of reference *S. epidermidis* ATCC 12228 were used to discover the minimal number of genomes, which could be identified (Khodaparast et al. 2016a).

4.2.4 Sequencing of *sesC* amplicons

The entire *sesC* gene was sequenced for five different *S. epidermidis* strains. Therefore, genomic DNA was extracted from each isolate using a 'Wizard genomic DNA purification kit' (Promega) with the addition of 30 µg lysostaphin (AMBI) $^{-1}$ in the lysis step. The sequencing was performed using sesC-F1, sesC-F2, sesC- F3, sesC-R1, and sesC-R2 primers (Table 4.1) on a ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. A protein homology analysis was performed using the BLASTP program (Altschul et al. 1990).

4.3 Results and Discussion

4.3.1 Identification of staphylococcal isolates

From the 438 clinical *Staphylococcus* isolates taken from patients, 175 isolates were identified as *S. epidermidis* and of the remaining isolates, 33 were identified as methicillin-resistant *Staphylococcus aureus* (MRSA), 50 isolates as methicillin-sensitive *Staphylococcus aureus* (MSSA). The 180 other isolates were CoNS including 43 *Staphylococcus haemolyticus,* 40 *Staphylococcus hominis,* 29 *Staphylococcus warneri,* 19 *Staphylococcus capitis*, 12 *Staphylococcus pasteuri*, 11

Staphylococcus lugdunensis strain, 9 *Staphylococcus saprophyticus*, 6 *Staphylococcus intermedius,* 4 *Staphylococcus carnosus,* 3 *Staphylococcus xylosous,* 3 *Staphylococcus simulans,* 1 *Staphylococcus pseudintermedius*. Table 4.2 shows the best matching NCBI strains as determined by mass spectrometry (WALDORF, Bruker). These strains all have different antibiotic susceptibility patterns (listed in the Table 4.4).

Table 4.2 List of strain-types achieved from the best rank of MALDI-TOF MS (Bruker)

Number of strains	Best match type-strains
n= 49	*Staphylococcus epidermidis* 10547 CHB
n= 32	*Staphylococcus epidermidis* DSM 3269
n= 26	*Staphylococcus epidermidis* 4b-r ESL
n= 24	*Staphylococcus epidermidis* 6b-s ESL
n= 18	*Staphylococcus epidermidis* ATCC 14990 T TH2
n= 11	*Staphylococcus epidermidis* CCM 4505
n= 4	*Staphylococcus epidermidis sk 135 hits*
n= 4	*Staphylococcus epidermidis BCM-HM p0060*
n= 3	*Staphylococcus epidermidis* LMG 10474
n= 3	*Staphylococcus epidermidis* ATCC 12228 THL
n= 1	*Staphylococcus epidermidis* JCM 2414

4.3.2 Exclusive presence of *sesC* in *S. epidermidis* isolates

Colony PCR on all staphylococcal strains using *sesC*-specific primers revealed that *sesC* was exclusively present in the isolates identified as *S. epidermidis,* while for the other strains no *sesC*-related PCR product could be obtained (Figure 4.1). Thus, using the *sesC* gene as a marker, we were able to easily distinguish *S. epidermidis* isolates from other species.

Recently, Thomas *et al*. reported 6 *sesC*-negative strains among 129 *S. epidermidis* isolates (Thomas et al. 2014). However, these *sesC*-negative strains proved to be *sesC*-positive on further examination (Robinson, D.A., Pers. Comm).

Noteworthy, another *S. epidermidis* surface gene, namely *sesI*, was earlier reported to be exclusively linked to invasive *S. epidermidis* strains. The *sesI* gene could not be detected among strains of the skin microbiota, while it was present in about 50% of the S. *epidermidis* cultures isolated from invasive infections (Söderquist et al. 2009).

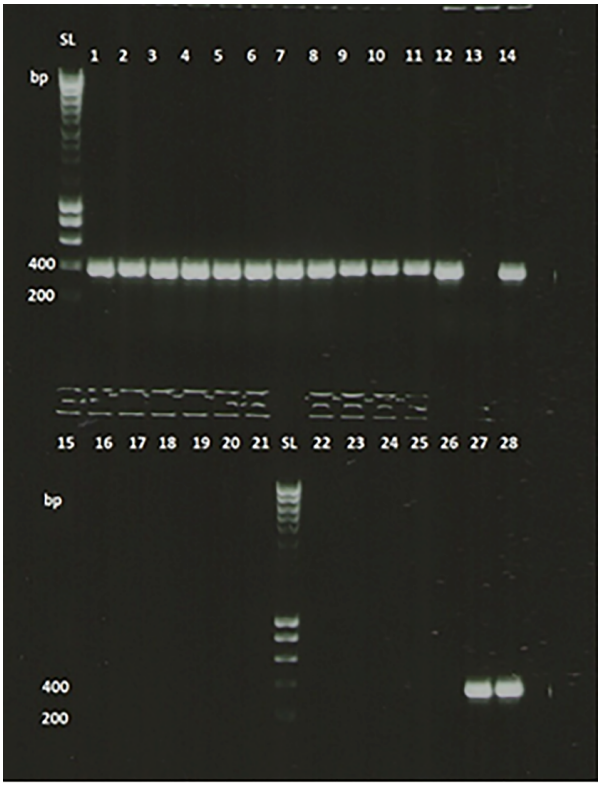

Figure 4.1 Example of colony PCR amplification using sesC-F1/R1 primer pair on different staphylococcal strains. Lanes 1-12, *S. epidermidis* isolates/strains; 13, negative control without template DNA; 14, positive control; 15, *S. aureus* MSSA; 16, *S. aureus* MRSA; 17-18, *S. hominis*; 19-20, *S. haemolyticus*; 21-22, *S. warneri*; 23-24, *S. capitis*; 25, *S. saprophyticus*; 26, *S. pasteuri*; 27-28, *S. epidermidis* ATCC 12228 and 10b. SL, Smart-ladder molecular size marker (Eurogentec)

4.3.3 Sequencing of *sesC*

Nucleotide sequencing of the *sesC* gene of 5 *S. epidermidis* isolates revealed that the nucleotide sequence of the homologous genes were 96% to 99% identical to *sesC* of *S. epidermidis* ATCC 12228 (Table 4.3). These results suggest that the *sesC* gene has become a conserved DNA sequence in all *S. epidermidis* strains, but absent in other staphylococcal species. Therefore, it can be used as a marker to screen *S. epidermidis* from other microorganisms.

PCR-based identification is an easier, faster and cheaper way for identification compared with those two techniques together, VITEK and MALDI-TOF. Of course, these systems are expensive and they are not available in every laboratory. To know a cheaper and faster technique like PCR-based would be helpful to the diagnosis of the early phase of infection and to prevent the severity of infection in the patient.

Taken together, *sesC* may be essential for viability and seems to be a unique genomic feature of this bacterial species. We demonstrated that a colony PCR assay based on the *sesC* gene might be a very useful tool for the reliable identification of *S. epidermidis* strains. PCR-based identification is an easier, faster and cheaper way for identification compared with alternative techniques, like VITEK and MALDI-TOF. These systems are expensive and thus not affordable for every laboratory, especially not in developing countries. The PCR-based identification will potentially improve the diagnosis of biomaterial-related infections and decrease the time to identification. Consequently, it would be helpful to the diagnosis of the early phase of infection, and to prevent the severity of infection in the patient.

Nevertheless, screening of more clinical isolates belonging to a broader range of staphylococcal strains, may be advisable to further validate the outcome of this study.

Table 4.3 Differences in amino acid sequence between SesC of *S. epidermidis* ATCC 12228 and of the *S. epidermidis* isolates S32, S146, 10b and S64, as deduced from DNA sequence analysis. (Substituted residues in SesC homologs are indicated in bold)

	SesC from				
Aa number	**ATTC 12228**	**S32**	**S146**	**10b**	**S64**
156	N	**E**	N	N	N
123	K	**N**	K	K	K
157	S	**R**	S	S	S
212	K	**N**	K	K	K
214	L	**P**	L	L	L
255	R	**H**	R	R	R
262	I	I	I	I	**L**
310	S	S	**P**	S	S
325	S	S	S	S	**A**
401	R	R	R	R	**Q**
554	K	K	K	**E**	K
613	S	S	S	**P**	S

Table 4.4. Patients information and antibiogram pattern of the strains used in this study.

	S name	strains	code	date	Column1	erythromycin	Clindamycin	Gentamicine	levofloxacine	tobramycine	Penicilline	vancomycine	amoxicilline	Rifampicin	Source
a1	st1	s.epidermidis	v-1004-42- hadan	12,04,12	*Staphylococcus epidermidis* 10547 CHB	R	S	S	S	R	S	S	S	S	exudate / pus (thoracotomy scar)
a2	st2	s.epidermidis	v-0704-52-abKI:n/st2 zw69P ID	12,04,12	*Staphylococcus epidermidis* 10547 CHB	R	R	S	S	R	R	S	R	R	biopsy / tissue (wound abdominal wall)
a3	st4	s.epidermidis	v-0704-53:kI/st2 ZW69R ID	12,04,12	*Staphylococcus epidermidis* DSM 3269	S	S	R	I	R	R	S	R	R	exudate / pus (abdomen)
a4	st8	s.epidermidis	v-2304-11: id /sta ZVHCC ID VERBEKE	24,04,12	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	S	S	S	S	S	ear
a5	st10	s.epidermidis	v-2204-20:ib/sta ZVUYN ID	24,04,12	*Staphylococcus epidermidis* DSM 3269	R	S	S	S	S	S	S	S	S	wondvocht/etter (vocht slipelen langs navelkatheter)
a6	st11	s.epidermidis	v-1204-82	24,04,12	*Staphylococcus epidermidis* DSM 3269	R	R	S	R	R	R	S	R	R	exudate / pus (hematoma)
a7	st12	s.epidermidis	v-1304-68:ID /sta ZTRNN ID	24,04,12	*Staphylococcus epidermidis* CCM 4505	S	S	S	S	S	S	S	S	S	exudate / pus (sternotomy)
a8	st13	s.epidermidis	v-1304-17:id/sta ZTRNM ID	24,04,12	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	S	S	S	S	S	exudate / pus (sore shoulder)
a9	st 14	s.epidermidis	v-2304-35:id/sta ZVHCP ID /BLOOD P	24,04,12	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	s	s	s	s	R	S	exudate / pus (left foot)
a10	st15	s.epidermidis	v-2304-36:id /sta ZVHCQ ID /BLOOD I	24,04,12	*Staphylococcus epidermidis* 10547 CHB	R	R	S	S	S	S	S	S	S	exudate / pus (upper left leg)
a11	st 16	s.epidermidis	v-2304-37:id /sta ZVHCR ID / BLOOD	24,04,12	*Staphylococcus epidermidis* CCM 4505	S	S	S	S	S	R	S	R	S	exudate / pus (under left arm)
a12	st17	s.epidermidis	v-2304-31: id /sta ZVHCJ ID	24,04,12	*Staphylococcus epidermidis* CCM 4505	R	S	S	S	S	S	S	S	S	exudate / pus (right shoulder)
a13	st18	s.epidermidis	v-2304-34 :id sta ZVHCP ID / BLOOD	24,04,12	*Staphylococcus epidermidis* CCM 4505	S	S	S	S	S	S	S	R	S	exudate / pus (left leg)
a14	st19	s.epidermidis	v-0802-61	16,2,12	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	S	R	S	R	S	exudate / pus (heel)
a15	st20	s.epidermidis	v-0802-66	16,2,12	*Staphylococcus epidermidis* DSM 3269	R	R	R	S	R	S	R	R	R	wound (finger)
a16	st21	s.epidermidis	v-080272	16,2,12	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	S	S	S	S	S	vagina
a17	st22	s.epidermidis	v-080280	16,2,12	*Staphylococcus epidermidis* CCM 4505	R	S	S	S	S	S	S	S	S	exudate / pus (under nose)
a18	st23	s.epidermidis	v-0802-35	16,2,12	*Staphylococcus epidermidis* DSM 3269	R	R	S	R	R	R	S	R	R	sternal wound
a19	st24	s.epidermidis	v-0802-53	16,2,12	*Staphylococcus epidermidis* CCM 4505	S	S	S	S	S	S	S	S	S	exudate / pus (insertion point) li groin
a20	st25	s.epidermidis	v-0702-89	16,2,12	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	R	S	S	S	wondvocht/etter (wonde stuit)
a21	st26	s.epidermidis	hc 0802-7	16,2,12	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	R	R	S	R	S	Pediatrics (white)
a22	st27	s.epidermidis	hc-0802-10	16,2,12	*Staphylococcus epidermidis* CCM 4505	R	S	S	S	S	S	S	S	S	anaerobic (set 1 DVC)
a23	st28	s.epidermidis	hc-0802-12	16,2,12	*Staphylococcus epidermidis* DSM 3269	R	R	R	R	R	R	S	R	R	aerobic set
a24	st29	s.epidermidis	hc-0802-28	16,2,12	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	S	S	S	S	S	S	Anaerobic (ctaheter)
a25	st30	s.epidermidis	hc-0802-17	16,2,12	*Staphylococcus epidermidis* CCM 4505	S	S	S	R	S	S	S	R	S	chatheter lumen
a26	st39	s.epidermidis	v-2404 32 ZVVZJ	1,05,2012	*Staphylococcus epidermidis* CCM 4505	R	R	S	R	R	R	S	R	R	exudate / pus (sternal wound)
a27	st40	s.epidermidis	V4-2304-3:STA ZYBTH ID	1,05,2012	*Staphylococcus epidermidis* 10547 CHB	S	S	S	I	S	R	S	S	S	blood catheter tip
a28	st41	s.epidermidis	V-2304-56/ab/sta ZYJRD ID	1,05,2012	*Staphylococcus epidermidis* CCM 4505	R	S	S	S	S	R	S	S	S	redon-drain tip
a29	st48	s.epidermidis	v-2304 59: ab-sta	1,05,2012	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	S	S	S	S	S	Blood
a30	st49	s.epidermidis	v8-1904-9: id-sta ZVWUV ID	1,05,2012	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	S	R	S	S	S	tissue bank
a31	st54	s.epidermidis	v-1704-16 ZTRVD	1,05,2012	*Staphylococcus epidermidis sk 135 hits*	R	R	R	R	R	R	S	R	R	biopsy / tissue (bottom plate thv fracture)
a32	st55	s.epidermidis	v-2204-3 ZVUYK	1,05,2012	*Staphylococcus epidermidis* 4b-r ESL	S	S	R	S	S	R	S	R	R	drain fluid link
a33	st56	s.epidermidis	v-2204-3 ZW19A	1,05,2012	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	S	S	S	S	Wound (ear)
a34	st60	s.epidermidis	V-2604 89 ZXBNY	22,05,2012	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	R	S	S	R	S	pus/exudate
a35	st62	s.epidermidis	V-27004 26 ZXBNK	22,05,2012	*Staphylococcus epidermidis* CCM 4505	R	S	S	S	S	R	S	R	S	exudate festering wound li chest
a36	st63	s.epidermidis	V-2604 101 ZXBNZ	22,05,2012	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	S	S	S	S	S	exudate / pus (toe)
a37	st64	s.epidermidis	V-2704 85 ZXBNN	22,05,2012	*Staphylococcus epidermidis sk 135 hits*	S	S	S	S	S	R	S	R	S	exudate / pus (abscess anal)
a38	st65	s.epidermidis	V_2704_44 ZXBNL	22,05,2012	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	S	R	S	R	S	catheter
a39	st77	s.epidermidis	V-0305 50	22,05,2012	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	S	R	S	R	S	exudate / pus (donor reBB)
a40	st83	s.epidermidis	V-1105-51	22,05,2012	*Staphylococcus epidermidis* DSM 3269	R	R	R	R	R	R	S	R	R	biopsy / tissue
a41	st85	s.epidermidis	V-110592	22,05,2012	*Staphylococcus epidermidis BCM-HM p0060*	R	R	S	S	S	R	S	S	S	biopsy / tissue (femur)
a42	st86	s.epidermidis	V-1605 53	22,05,2012	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	S	S	R	S	S	S	biopsy / tissue (on the sacrum)
a43	st88	s.epidermidis	V4-1106_14	15,06,12	*Staphylococcus epidermidis* LMG 10474	R	R	S	R	R	R	S	R	R	Wound (naile)
a44	st89	s.epidermidis	V4-1206-11	15,06,12	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	S	R	S	R	biopsy/tissue (left leg)
a45	st98	s.epidermidis	V-3005-41	15,06,12	*Staphylococcus epidermidis* ICM 2414	S	S	S	S	S	R	S	R	S	exudate / pus (gluteus)
a46	st100	s.epidermidis	V-3005-15	15,06,12	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	S	S	S	S	S	exudate / pus (tibia pin)
a47	St102	s.epidermidis	V-3005-80	15,06,12	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	S	S	S	S	S	biopsy / tissue (tibia)
a48	St103	s.epidermidis	V-3005-81	15,06,12	*Staphylococcus epidermidis* DSM 3269	R	R	R	S	R	R	S	R	R	exudate / pus (knee)
a49	St104	s.epidermidis	V-3005-95	15,06,12	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	S	S	R	S	S	S	exudate / pus (sternal wound)
a50	St121	s.epidermidis	V-4232-109	01,03,12	*Staphylococcus epidermidis* 4b-r ESL	R	R	S	S	R	R	S	S	R	biopsy / tissue (distal femur)
a51	St122	s.epidermidis	hc-0703_10	01,03,12	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	S	S	R	S	R	S	drain fluid link
a52	St123	s.epidermidis	hc-0703_11	01,03,12	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	R	S	S	S	Catheter
a53	St124	s.epidermidis	hc-0703_15	01,03,12	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	S	S	S	S	S	S	Blood
a54	St125	s.epidermidis	hc-0703_26	01,03,12	*Staphylococcus epidermidis* DSM 3269	R	R	R	S	R	R	S	S	S	exudate / pus (eye)
a55	St126	s.epidermidis	V-0292-15	01,03,12	*Staphylococcus epidermidis* DSM 3269	S	S	S	R	S	S	S	R	S	Biopsy/tissue (mouth)
a56	St127	s.epidermidis	hc-0159_64	01,03,12	*Staphylococcus epidermidis* DSM 3269	R	R	S	S	S	R	S	R	R	Catheter
a57	St138	s.epidermidis	V-0292-12	01,03,12	*Staphylococcus epidermidis* 4b-r ESL	S	R	S	R	R	R	S	R	R	Catheter
a58	St139	s.epidermidis	hc-0159_65	03,02,12	*Staphylococcus epidermidis* 10547 CHB	R	S	S	R	R	S	S	S	S	Wound (chest)
a59	St140	s.epidermidis	V-0285-12	03,02,12	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	R	S	S	R	S	Wound (right hand)
a60	St142	s.epidermidis	V-0285-13	03,02,12	*Staphylococcus epidermidis* 10547 CHB	R	R	S	R	S	R	S	S	S	vagina
a61	S12	s.epidermidis	V-0285-16	03,02,12	*Staphylococcus epidermidis sk 135 hits*	R	R	S	s	S	S	S	R	R	blood
a62	S13	s.epidermidis	V-0285-19	03,02,12	*Staphylococcus epidermidis* 10547 CHB	R	S	S	S	S	R	S	S	S	catheter leumen
a63	S15	s.epidermidis	V-0285-20	03,02,12	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	S	S	S	S	s	exudate / pus (gum)
a64	S32	s.epidermidis	V-0285-21	03,02,12	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	S	S	R	S	S	s	blood
a65	S36	s.epidermidis	V-0285-22	03,02,12	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	S	S	R	S	S	S	urine
a66	S39	s.epidermidis	V-0285-28	03,02,12	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	S	S	S	S	S	urine
a67	S42	s.epidermidis	V-0285-24	03,02,12	*Staphylococcus epidermidis* 10547 CHB	R	S	S	S	R	R	S	R	S	exudate / pus (hematoma)

a68	S44	s,epidermidis	V-0285-16	03,02,12	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	S	S	S	S	exudate / pus (under left arm)
a69	S45	s,epidermidis	V-0285-97	03,02,12	*Staphylococcus epidermidis* 10547 CHB	R	R	R	S	S	R	S	R	R	wondvocht/etter (wonde stuit)
a70	S46	s,epidermidis	hc-0034-2	15,01,12	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	S	S	S	S	R	biopsy / tissue (bottom plate thv fracture)
a71	S47	s,epidermidis	hc-0034-3	15,01,12	*Staphylococcus epidermidis* DSM 3269	S	R	S	S	S	S	S	R	R	exudate / pus (donor reBB)
a72	S48	s,epidermidis	hc-0034-6	15,01,12	*Staphylococcus epidermidis* DSM 3269	R	S	S	S	S	S	S	S	S	biopsy / tissue (tibia)
a73	S49	s,epidermidis	hc-0034-7	15,01,12	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	S	S	S	R	blood
a74	S50	s,epidermidis	hc-0034-11	15,01,12	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	R	S	S	S	S	exudate / pus (neck)
a75	S51	s,epidermidis	V-0123_34	15,01,12	*Staphylococcus epidermidis* DSM 3269	R	S	S	S	S	R	S	S	S	mouth
a76	S52	s,epidermidis	V-0123_35	15,01,12	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	S	S	S	S	S	exudate / pus (lip)
a77	S53	s,epidermidis	?	15,01,12	*Staphylococcus epidermidis* 10547 CHB	R	S	S	s	S	R	S	S	S	?
a78	S54	s,epidermidis	?	15,01,12	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	s	S	R	S	S	R	?
a79	S55	s,epidermidis	?	15,01,12	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	S	S	S	S	S	?
a80	S56	s,epidermidis	V-0123_13	15,01,12	*Staphylococcus epidermidis* 10547 CHB	S	R	S	S	S	S	S	S	S	urine
a81	S57	s,epidermidis	V-0123_40	15,01,12	*Staphylococcus epidermidis* 10547 CHB	R	S	S	S	R	S	S	R	S	exudate / pus (face)
a82	S58	s,epidermidis	?	15,01,12	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	R	S	S	S	?
a83	S59	s,epidermidis	?	02,01,12	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	S	S	S	R	S	?
a84	S60	s,epidermidis	?	02,01,12	*Staphylococcus epidermidis* 10547 CHB	R	S	S	S	S	R	S	S	S	?
a85	S61	s,epidermidis	?	02,01,12	*Staphylococcus epidermidis BCM-HM p0060*	S	S	S	S	S	S	S	R	R	?
a86	S62	s,epidermidis	?	02,01,12	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	S	S	S	S	S	?
a87	S63	s,epidermidis	V-0123_12	02,01,12	*Staphylococcus epidermidis* LMG 10474	S	S	S	S	S	R	S	R	S	vagina
a88	S64	s,epidermidis	V-0123_37	02,01,12	*Staphylococcus epidermidis* LMG 10474	R	R	S	S	S	S	S	R	S	biopsy / tissue (knee)
a89	S65	s,epidermidis	V-0123_25	02,01,12	*Staphylococcus epidermidis* 10547 CHB	R	R	S	S	S	R	S	S	S	exudate / pus (face)
a90	S66	s,epidermidis	hc-093-15	02,01,12	*Staphylococcus epidermidis* ATCC 12228 THL	S	S	s	S	R	R	S	S	S	wound (hand)
a91	S67	s,epidermidis	V-0123_41	02,01,12	*Staphylococcus epidermidis* 4b-r ESL	S	R	S	S	S	R	S	S	S	exudate / pus (nose)
a92	S68	s,epidermidis	V-0123_6	02,01,12	*Staphylococcus epidermidis sk 135 hits*	S	S	S	S	S	S	S	S	R	catheter leumen
a93	S69	s,epidermidis	V-0123_4	02,01,12	*Staphylococcus epidermidis* 10547 CHB	R	S	S	S	S	S	S	S	S	blood
a94	S70	s,epidermidis	V-0123_12	02,01,12	*Staphylococcus epidermidis* 10547 CHB	S	S	s	S	S	S	S	S	R	exudate / pus (face)
a95	S71	s,epidermidis	hc-0094-2	02,01,12	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	R	S	S	R	exudate / pus (left S-neck)
a96	S72	s,epidermidis	hc-0094-3	02,01,12	*Staphylococcus epidermidis* DSM 3269	S	R	s	R	S	S	S	R	R	acne (neck)
a97	S73	s,epidermidis	hc-0094-9	02,01,12	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	S	S	S	S	S	exudate / pus (eyes)
a98	S74	s,epidermidis	?	02,01,12	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	S	S	S	R	?
a99	S75	s,epidermidis	v-0343-32 ZW093	02,01,12	*Staphylococcus epidermidis* 4b-r ESL	R	S	S	S	R	S	S	S	R	Wound (chest)
a100	S76	s,epidermidis	v-0343-29	03,12,2011	*Staphylococcus epidermidis* DSM 3269	S	S	s	S	S	S	S	S	S	blood
a101	S77	s,epidermidis	v-0343-18 WZH	03,12,2011	*Staphylococcus epidermidis* 4b-r ESL	S	S	s	S	S	R	S	S	S	biopsy / tissue (arm)
a102	S78	s,epidermidis	v-0343-3 XZWW	03,12,2011	*Staphylococcus epidermidis* 10547 CHB	R	R	S	S	S	R	S	R	S	biopsy / tissue (femur)
a103	S79	s,epidermidis	v-0343-38cdf	03,12,2011	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	S	R	S	S	S	exudate / pus (ear)
a104	S80	s,epidermidis	v-0343-13 ZWX	03,12,2011	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	R	R	S	S	S	urine
a105	S81	s,epidermidis	v-0343-23	03,12,2011	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	S	S	S	S	catheter
a106	S82	s,epidermidis	v-0343-1 bxv	03,12,2011	*Staphylococcus epidermidis* 10547 CHB	R	R	S	S	R	S	S	S	S	vagina
a107	S83	s,epidermidis	v-00200-6	03,12,2011	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	S	S	S	R	mouth
a108	S84	s,epidermidis	v-00200-20	03,12,2011	*Staphylococcus epidermidis BCM-HM p0060*	S	S	S	S	S	R	S	R	R	biopsy (newborn skin)
a109	S85	s,epidermidis	v-00200-90	03,12,2011	*Staphylococcus epidermidis* DSM 3269	S	S	S	S	R	S	S	R	S	biopsy / tissue (neck)
a110	S86	s,epidermidis	v-00200-12	03,12,2011	*Staphylococcus epidermidis* DSM 3269	R	S	S	S	S	R	S	S	S	wound (right leg)
a111	S87	s,epidermidis	v-00200-82	03,12,2011	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	S	S	S	S	exudate / pus (Anus)
a112	S88	s,epidermidis	v-00200-1	03,12,2011	*Staphylococcus epidermidis* ATCC 12228 THL	S	R	S	R	R	R	R	R	R	catheter
a113	S89	s,epidermidis	v-0321-2	03,12,2011	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	R	S	S	S	Blood
a114	S90	s,epidermidis	hc-4002-5	03,12,2011	*Staphylococcus epidermidis* 10547 CHB	R	R	S	S	S	S	S	S	S	exudate / pus (face)
a115	S91	s,epidermidis	hc-4002-6	03,12,2011	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	S	S	R	S	S	S	exudate / pus (Iris)
a116	S92	s,epidermidis	hc-4002-32	16,12,11	*Staphylococcus epidermidis* DSM 3269	R	R	S	S	S	R	S	S	S	biopsy / tissue (femur)
a117	S93	s,epidermidis	V-5443_13	16,12,11	*Staphylococcus epidermidis* DSM 3269	S	S	S	I	R	R	S	S	R	biopsy/tissue (left leg)
a118	S94	s,epidermidis	V-5443_14	16,12,11	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	R	S	S	R	exudate / pus (gluteus)
a119	S95	s,epidermidis	V-5443_15	16,12,11	*Staphylococcus epidermidis* ATCC 14990 T TH2	R	S	R	S	S	R	R	R	R	exudate / pus (Knee)
a120	S96	s,epidermidis	hc-0932-3	16,12,11	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	I	S	S	S	R	S	biopsy / tissue (distal femur)
a121	S97	s,epidermidis	V-5443-1	16,12,11	*Staphylococcus epidermidis* 4b-r ESL	S	R	S	S	S	R	S	R	S	exudate / pus (tongue)
a122	S98	s,epidermidis	V-5443_14	16,12,11	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	S	S	S	R	S	exudate / pus (Nipple)
a123	S99	s,epidermidis	V-5443_16	16,12,11	*Staphylococcus epidermidis* 6b-s ESL	R	S	S	S	S	S	S	S	S	urine
a124	S100	s,epidermidis	V-5443_15	16,12,11	*Staphylococcus epidermidis* 10547 CHB	R	R	S	R	S	R	S	R	R	infant cord
a125	S101	s,epidermidis	hc-200-2 zwx	16,12,11	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	S	S	R	S	wound (nipple)
a126	S102	s,epidermidis	hc-200-64x	16,12,11	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	S	S	R	S	Wound (chest)
a127	S103	s,epidermidis	hc-200-76 ZXW	16,12,11	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	R	S	S	S	R	S	S	S	exudate / pus (ear)
a128	S104	s,epidermidis	V-200-21 JZWW	16,12,11	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	R	R	R	S	R	R	blood
a129	S105	s,epidermidis	hc-354-13	16,12,11	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	I	R	R	S	R	S	exudate / pus (nose)
a130	S106	s,epidermidis	hc-354-14	16,12,11	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	R	S	S	S	catheter
a131	S107	s,epidermidis	hc-354-15	16,12,11	*Staphylococcus epidermidis* 10547 CHB	R	S	S	S	S	S	S	S	S	exudate / pus (eyelid)
a132	S108	s,epidermidis	hc-354-16	16,12,11	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	S	S	S	S	urine
a133	S109	s,epidermidis	V-2006-32 mlk	16,12,11	*Staphylococcus epidermidis* 6b-s ESL	R	R	S	R	R	R	R	R	R	blood
a134	S110	s,epidermidis	V-2006-34	16,12,11	*Staphylococcus epidermidis* 6b-s ESL	R	S	S	S	S	S	S	S	S	urine
a135	S111	s,epidermidis	V-2006-46	04,11,2011	*Staphylococcus epidermidis* 4b-r ESL	R	R	S	S	S	S	S	R	S	urine
a136	S112	s,epidermidis	V-2006-47	04,11,2011	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	S	S	S	S	S	S	Catheter
a137	S113	s,epidermidis	V-2006-56	04,11,2011	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	R	S	S	S	S	S	exudate / pus (left leg)

a138	S114	s.epidermidis	V-1930-11	04.11.2011	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	S	S	S	S	S	s	s	s	exudate / pus (eye)
a139	S115	s.epidermidis	V-1930-11 szxc	04.11.2011	*Staphylococcus epidermidis* 4b-r ESL	R	S	S	I	R	S	S	S	S	S	S	acne (face)
a140	S116	s.epidermidis	V-1930-15	04.11.2011	*Staphylococcus epidermidis* 6b-s ESL	s	S	S	S	S	R	S	S	S	S	R	biopsy / tissue (wound anus)
a141	S117	s.epidermidis	V-654-54	04.11.2011	*Staphylococcus epidermidis* 4b-r ESL	S	S	S	S	S	S	S	S	S	S	S	Respiratory tract
a142	S118	s.epidermidis	hc-120-30	04.11.2011	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	S	S	S	R	S	S	exudate / pus (sternotomy)
a143	S119	s.epidermidis	V-654-26	04.11.2011	*Staphylococcus epidermidis* 10547 CHB	S	S	s	S	R	S	S	S	S	S	S	vagina
a144	S120	s.epidermidis	V-654-21	04.11.2011	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	S	R	S	S	S	S	exudate / pus (nose)
a145	S121	s.epidermidis	V-654-13	04.11.2011	*Staphylococcus epidermidis* ATCC 14990 T TH2	R	S	S	S	S	S	S	S	S	S	S	blood
a146	S122	s.epidermidis	V-654-12	04.11.2011	*Staphylococcus epidermidis* 10547 CHB	R	R	R	R	R	R	R	R	R	R	R	urine
a147	S123	s.epidermidis	hc-0056-61 nwb	04.11.2011	*Staphylococcus epidermidis BCM-HM-p0060*	S	S	S	S	S	S	S	S	S	s	s	Inflammation thumb
a148	S124	s.epidermidis	hc-0056-32	04.11.2011	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	S	S	S	R	S	S	exudate / pus (navel)
a149	S125	s.epidermidis	hc-0056-24	04.11.2011	*Staphylococcus epidermidis* 6b-s ESL	S	R	S	S	R	S	S	S	S	S	S	injection (elbow)
a150	S126	s.epidermidis	hc-0056-21	04.11.2011	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	S	S	S	R	S	S	Respiratory tract
a151	S127	s.epidermidis	hc-0056-16	04.11.2011	*Staphylococcus epidermidis* 6b-s ESL	S	R	S	S	S	S	S	S	S	S	S	biopsy / tissue (shoulder)
a152	S128	s.epidermidis	V-546-12	17.11.11	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	R	S	S	S	S	R	S	S	exudate / pus (nose)
a153	S129	s.epidermidis	V-546-12 hxc	17.11.11	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	R	S	S	S	S	S	S	wound (back)
a154	S130	s.epidermidis	V-546-13	17.11.11	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	S	S	S	S	S	S	biopsy (left leg)
a155	S131	s.epidermidis	V-546-18	17.11.11	*Staphylococcus epidermidis* 10547 CHB	R	S	S	S	S	S	S	S	S	S	S	urine
a156	S132	s.epidermidis	V-546-32	17.11.11	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	R	S	S	S	S	S	Biopsy/tissue (lip)
a157	S133	s.epidermidis	V-451-41	17.11.11	*Staphylococcus epidermidis* 10547 CHB	R	S	S	S	S	S	S	S	S	S	S	exudate / pus (head)
a158	S134	s.epidermidis	V-451-46	17.11.11	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	R	S	S	S	S	S	S	S	exudate / pus (hemorrhoid)
a159	S135	s.epidermidis	V-451-32	12.04.12	*Staphylococcus epidermidis* ATCC 14990 T TH2	R	R	S	R	R	R	R	R	R	R	R	catheter
a160	S136	s.epidermidis	V-451-15	12.04.12	*Staphylococcus epidermidis* 6b-s ESL	S	R	S	S	S	R	S	S	S	S	S	biopsy/ tissue (under armpit)
a161	S137	s.epidermidis	V-451-14 hndj	12.04.12	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	S	S	S	S	S	S	Respiratory tract
a162	S138	s.epidermidis	v-0136-10mlf	17.11.11	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	S	R	S	R	S	S	urine
a163	S139	s.epidermidis	v-0136-3	17.11.11	*Staphylococcus epidermidis* ATCC 12228 THL	R	s	S	S	S	S	S	s	S	S	S	biopsy / tissue (wound abdominal)
a164	S140	s.epidermidis	v-0136-4zxw	17.11.11	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	S	S	S	S	S	S	exudate / pus (hematoma)
a165	S141	s.epidermidis	v-0136-5	17.11.11	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	R	S	S	S	S	R	S	S	exudate / pus (left leg)
a166	S142	s.epidermidis	hc-0054-12	17.11.11	*Staphylococcus epidermidis* 10547 CHB	S	S	S	R	S	S	S	S	S	S	S	tissue bank
a167	S143	s.epidermidis	hc-0054-16	17.11.11	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	S	S	S	R	S	S	pus/exudate(ear)
a168	S144	s.epidermidis	hc-0054-15	17.11.11	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	s	S	R	S	R	S	S	blood
a169	S145	s.epidermidis		23.12.2009	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	S	S	s	S	S	S	?
a170	S146	s.epidermidis		23.12.2009	*Staphylococcus epidermidis* 6b-s ESL	R	R	R	R	R	R	R	S	R	R	R	?
a171	S147	s.epidermidis		23.12.2009	*Staphylococcus epidermidis* 6b-s ESL	S	S	S	S	S	S	R	S	S	S	S	?
a172	S148	s.epidermidis		23.12.2009	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	I	R	R	S	S	R	S	S	?
a173	S149	s.epidermidis		23.12.2009	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	R	S	S	S	S	S	?
a174	S150	s.epidermidis		23.12.2009	*Staphylococcus epidermidis* 10547 CHB	S	S	S	S	S	R	S	S	S	S	S	?
a175	S151	s.epidermidis		23.12.2009	*Staphylococcus epidermidis* ATCC 14990 T TH2	S	S	S	S	S	S	S	S	S	S	S	?
		s.epidermidis	ATCC12228			S	S	s					S				References
		s.epidermidis	10b			R	R	s					S				patient
		s.epidermidis	RP62A			S	S	s					S				References
		s.epidermidis	1457			R		S					S				References
		s.epidermidis	TU3298					S				R	S				References

CHAPTER V: The Possible Role of *Staphylococcus epidermidis* LPxTG Surface Protein SesC in Biofilm Formation

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RESEARCH ARTICLE

The Possible Role of *Staphylococcus epidermidis* LPxTG Surface Protein SesC in Biofilm Formation

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Abstract

Staphylococcus epidermidis is the most common cause of device-associated infections. It has been shown that active and passive immunization in an animal model against protein SesC significantly reduces *S. epidermidis* biofilm-associated infections. In order to elucidate its role, knock-out of *sesC* or isolation of *S. epidermidis sesC*-negative mutants were attempted, however, without success. As an alternative strategy, *sesC* was introduced into *Staphylococcus aureus* 8325–4 and its isogenic *icaADBC* and *srtA* mutants, into the clinical methicillin-sensitive *S. aureus* isolate MSSA4 and the MRSA *S. aureus* isolate BH1CC, which all lack *sesC*. Transformation of these strains with *sesC* i) changed the biofilm phenotype of strains 8325–4 and MSSA4 from PIA-dependent to proteinaceous even though PIA synthesis was not affected, ii) converted the non-biofilm-forming strain 8325–4 *ica*::*tet* to a proteinaceous biofilm-forming strain, iii) impaired PIA-dependent biofilm formation by 8325–4 *srtA*::*tet*, iv) had no impact on protein-mediated biofilm formation of BH1CC and v) increased *in vivo* catheter and organ colonization by strain 8325–4. Furthermore, treatment with anti-SesC antibodies significantly reduced *in vitro* biofilm formation and *in vivo* colonization by these transformants expressing *sesC*. These findings strongly suggest that SesC is involved in *S. epidermidis* attachment to and subsequent biofilm formation on a substrate.

5.1 Introduction

Of all coagulase-negative staphylococci, *S. epidermidis* is the most common cause of infections associated with catheters and other indwelling medical devices (O'Gara & Humphreys 2001; Otto 2009). It is a permanent and ubiquitous colonizer of human skin, can easily contaminate the medical devices during insertion, and subsequently form a biofilm (Otto 2009; Uçkay et al. 2009). The capacity to form a biofilm is considered as one of the major virulence factors of this bacterial species (Raad et al. 1998; Götz 2002). Staphylococcal biofilms develop via a multifactorial process, which may differ between species and strains. Nevertheless, most of the factors involved are analogous in *S. epidermidis* and *S. aureus* and have a similar function in biofilm formation (O'Gara & Humphreys 2001; Otto 2009; Uçkay et al. 2009).

Up to now, based on extracellular matrix macromolecules constituting the biofilm, three mechanisms of biofilm formation in staphylococci are identified (Rawlinson et al. 2011). Production of polysaccharide intercellular adhesin [PIA, also called poly-*N*-acetylglucosamine (PNAG)] was the first and for a long time, the only mechanism of biofilm formation identified (O'Gara 2007; Cheung & Otto 2010). Further studies showed the existence of other PIA- or *ica*-independent mechanisms in *S. aureus* and *S. epidermidis*. Based on *in vivo* and *in vitro* studies, the proteinaceous biofilm formation was identified. In this case, the cell-surface and cell-cell attachment is based on proteins (Fitzpatrick et al. 2005; Hennig et al. 2007). More recently, a third mechanism based on extracellular DNA (eDNA) constituting a cell-to-cell or cell-to-substratum connecting component was recognized. This eDNA originates from autolysis (Rice et al. 2007; Büttner et al. 2015).

It has been shown that staphylococcal surface proteins such as accumulation-associated protein (Aap), biofilm-associated proteins (Bap, and Bap homologue Bhp), extracellular matrix-binding protein (Embp), fibronectin- or fibrinogen-binding proteins (FnBPA, FnBPB and Fbe/SdrG), and the major autolysin (AtlE) mediate the formation of the network of multilayered cell clusters and filamentous proteins, and thus play an important role in the biofilm accumulation phase (O'Gara 2007; Hennig et al. 2007; Christner et al. 2010; Houston et al. 2011). In *S. epidermidis* and *S. aureus,* LPxTG motif-containing proteins which covalently link to the cell wall via sortase activity, are determinants in the pathogenesis of device-related infections (O'Gara 2007).

Through unknown or not well-characterized mechanisms such as insertion and excision of the insertion sequence IS*256* at specific hot spots of the *S. epidermidis icaA* and *icaC* genes,

PIA/PNAG production, biofilm formation and biofilm phenotype may be phase variable, allowing to switch from PIA-dependent to proteinaceous phenotype (Ziebuhr et al. 1999) (Conlon et al. 2002). In 2001, Knobloch *et al*. reported that NaCl affects biofilm formation through the activation of the *sigB* operon, an important regulator of the *ica* operon, and thus can be used to distinguish *ica*-dependent from *ica*-independent biofilm formation (Fitzpatrick et al. 2005; Conlon et al. 2002; Knobloch et al. 2001). By using dispersing agents such as sodium metaperiodate (SM), proteinase K (PK) and DNase I, the chemical composition of the biofilm extracellular polymeric substance can be determined and one can discriminate between PIA-dependent, proteinaceous and eDNA-based biofilms (Fitzpatrick et al. 2005; Pozzi et al. 2012).

So far, the roles of 5 *S. epidermidis* LPxTG proteins (Aap, Bhp, SdrF, SdrG, SesI) in the pathogenesis of *S. epidermidis* infections and biofilm formation have been studied (Davis et al. 2001; Bowden et al. 2005; Arrecubieta et al. 2009). We focused our research on the LPxTG motif-containing *S. epidermidis* surface protein SesC, a 676-amino acid (aa) protein with a predicted molecular mass of 75 kDa. The cytoplasmic precursor of SesC contains a 35-aa N-terminal signal peptide, required for Sec-dependent secretion and is cleaved off by the signal peptidase. The 37-aa C-terminal LPxTG-sorting signal is recognized by sortase, which will cleave the bond between the Thr and Gly and thereafter covalently link the 608-aa (68 kDa) remaining protein to the peptidoglycan layer. Using antibodies against the mature domain of the SesC protein, we were able to reduce *S. epidermidis* biofilm formation *in vitro* (Shahrooei et al. 2009). In addition, active and passive immunization against SesC could significantly reduce their biofilm formation on catheter fragments in animal models of subcutaneous and intravascular catheter infection (Shahrooei et al. 2009). However, the involvement and exact function of SesC in *S. epidermidis* biofilm formation have remained unknown, so far. In order to elucidate its role, knockout of *sesC* or isolation of *S. epidermidis sesC*-negative mutants were attempted, however without success. Therefore, as an alternative strategy *sesC* was introduced into *S. aureus* strains and the effect of *sesC* expression in biofilm formation by these host strains was studied. However, using this strategy, we should be careful with extrapolating conclusions in *S. epidermidis*, since they are different staphylococci.

5.2 Materials and Methods

5.2.1 Bacterial strains, plasmids and media

Cloning experiments were performed in *Escherichia coli* DH5α competent cells (Invitrogen). *E. coli* DH5α transformants were grown in Lysogeny Broth (LB) or on LB agar at 37°C supplemented with ampicillin (100 μg/), as all plasmids used in this study (Table 5.1) contain an ampicillin resistance (*bla*) gene. All *Staphylococcus* strains (Table 5.1) were grown in brain heart infusion (BHI) medium or agar, and for biofilm formation assays also in BHI medium supplemented with 4% NaCl (BHI-NaCl) or 1% glucose (BHI-glucose). Bacterial CFU counting was done on Tryptone Soya agar (TSA, Oxoid) or blood agar plates (BD Biosciences). Whenever required, growth media were supplemented with appropriate antibiotics as follows: chloramphenicol at 10 μg/, erythromycin at 10 μg/ and tetracycline at 5 μg/. Species identification and antibiograms for all clinical isolates were performed using a VITEK® 2 automated system (bioMérieux).

Table 5.1. *Staphylococcus* strains and plasmids used in this study.

Strains	Characteristic(s)	Reference
- S. epidermidis		
10b	Clinical isolate	(Van Wijngaerden et al. 1999)
- S. aureus		
RN4220	Restriction-negative derivative of 8325-4	(Kreiswirth BN, et al. 1983)
8325-4	NCTC8325 cured of prophages. 11-bp deletion in *rsbU*.	(Fitzpatrick et al. 2005)
BH1CC	MRSA clinical isolate. Biofilm positive. SCCmec type II, ST type 8, clonal complex 8.	(Fitzpatrick et al. 2005)
MSSA4	Clinical isolate	This study
8325-4 *ica::tet*	*icaADBC*::*tet*; isogenic mutant of 8325-4	(Fitzpatrick et al. 2005)
8325-4 *srtA::tet*	*srtA*::*tet*; isogenic mutant of 8325-4	(O'Neill et al. 2008)
BH1CC *ica*::*tet*	*icaADBC*::*tet*; isogenic mutant of BH1CC	(Fitzpatrick et al. 2005)
BH1CC *srtA*::*tet*	*srtA*::*tet*; isogenic mutant of BH1CC	(O'Neill et al. 2008)
Plasmids		
pCN68	*E. coli-Staphylococcus* shuttle vector	(Charpentier et al. 2004)
pSRsrtA5	*E. coli-Staphylococcus* shuttle vector	(O'Neill et al. 2008)
pET11c	*E. coli* vector	(Stratagene, La Jolla, CA)

5.2.2 Producing polyclonal antibody against SesC

5.2.2.1 Construction and purification of his-SesC protein

A truncation of the *sesC* gene with a sequence coding for a C-terminal His6 tag and incorporated flanking *Nhe*I and *Bam*HI (Table 5.2) sites, was designed and cloned in pET11C vector (Table 5.1) and transformed into *E. coli* BL21 (DE3). Protein was purified as described previously (Shahrooei et al. 2012). Briefly, *E. coli* BL2 pET11C-his-sesC was grown in 1 L Luria-Bertani (LB) broth with 100 µg/ ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C, overnight with shaking. To purify whole proteins, cells were harvested by centrifugation (4,000 rpm, 10 min, 4°C) and resuspended in 25 lysis buffer (PBS pH 7.5, 1 mM β-mercaptoethanol plus 1 tablet protease inhibitor Mini, EDTA-free), French pressed and stirred in 4°C with adding DNaseI. To get rid of aggregation, we kept the proteins on ice and filtered them before use.

Before the actual purification and sample injection into the AKTA, the His column was stripped, charged, and blanked. The purification was done by program in the AKTA Xpress. The purified proteins were collected from different tubes and the combined fractions were kept at 4 ºC. Since the buffer contained imidazole, the samples were dialyzed against PBS.

5.2.2.2 Immunization of rabbits and producing polyclonal antibody against SesC

Immunization was done during 10 days with two injections intraperitoneally. The first injection was administered with a mixture of SesC protein and Complete Freund's adjuvant (CFA; Sigma), and a booster injection was performed after titration of the antibody (ELISA) with protein and Incomplete Freund's adjuvant (IFA; Sigma) at day 10. Then from the serum, the total immunoglobulin Gs (IgGs) against rSesC (anti-rSesC) were purified by absorption to a protein G column (GE Healthcare) according to the manufacturer's instructions.

5.2.3 Cloning and expression of *S. epidermidis sesC* and *sesK* genes in *S. aureus* strains

The coding regions of *S. epidermidis sesC* (SE2232, Gene ID 1056520) and *sesK* (SE1501, Gene ID 1056680), were amplified using *sesC*- and *sesK*-specific primers (Table 5.2) containing additionally a *Sal*I or *Sma*I restriction site for cloning purposes. Genomic DNA (gDNA) of biofilm-forming *S. epidermidis* strain 10b, a clinical isolate (Van Wijngaerden et al. 1999), was used as a template. The amplicons were ligated into *Sal*I/*Sma*I-digested pCN68 *E. coli* - *Staphylococcus* shuttle vectors (Charpentier et al. 2004) yielding pCN*sesC*

and pCN*sesK*. In this plasmid, P*blaZ* is the promoter. It is a highly active constitutive promoter; erythromycin was used as the selection marker. All recombinant plasmids were replicated in *E. coli* DH5α. Correctness of cloning was confirmed by restriction enzyme digestion, PCR, and nucleotide sequence analysis of the insert. Plasmids harvested from *E. coli* were first electroporated into the restriction-deficient *S. aureus* strain RN4220 and subsequently into other *S. aureus* strains. Presence and expression of *sesC*, *sesK*, *sasF* (Gene ID: 5775591), and *icaA* (Gene ID: 5776135) in transformed strains were evaluated using gel-based reverse transcription-PCR (RT-PCR) and Western blotting assays. Plasmid, gDNA and RNA isolation from bacterial strains and cDNA synthesis were performed as previously described (Shahrooei et al. 2012).

Table 5.2. Primers used in this study

Name	Sequence (5' → 3')	RE site
sesCF	AT*GTCGAC*TTTATTAAAGGAGT**ATG**TGTAAATG	*Sal*I
sesCR	AT*CCCGGG*TGATGATGC*CTA*TTACTATATATAA	*Sma*I
sesKF	AT*GTCGAC*GACCTCTTAACTAATT**ATG**TTATG	*Sal*I
sesKR	AT*CCCGGG*TCTCGTTATTTTCACTCAAATATC	*Sma*I
rsesc354Fwd	ACGT*GCTAGC*GCAGATTCAGAAAGTACATC	*Nhe*I
rsesc354hisRev	ATGC*GGATCC*TAGTGATGGTGATGGTGATGATCAGCTGTAGCTGTTCC	*BamH*I

(Underlined sequences: restriction sites; bold sequence: start codon, blue His codons)

5.2.4 Biofilm formation assay

The amount of biofilm formed by the different strains was determined using a semi-quantitative adherence assay in 96-well polystyrene microtiter plates (BD Biosciences) as previously described (Shahrooei et al. 2009; Shahrooei et al. 2012). Briefly, 20 µl of stock cultures were inoculated into 5 (selective) BHI medium and grown to the end-exponential growth phase in a shaking incubator at 37°C. Cultures were subsequently diluted to an OD_{600} of 0.005 ($5x10^6$ CFU/) in fresh BHI medium supplemented or not with 4% NaCl or 1% glucose. 200 µl of the diluted cultures of bacteria were pipetted into sterile 96-well polystyrene microtiter plates and incubated overnight at 37°C without shaking.

After incubation, the wells were rinsed 3 times with phosphate-buffered saline (PBS) and dried afterwards. The adhered material was stained with 200 µl of a 1% (w/v) crystal violet (Sigma) solution for 10 min, and subsequently, the wells were washed 3 times with water and again dried. For quantification, 160 µl of 30% (v/v) acetic acid solution was added to

each well to dissolve the crystal violet. The OD_{595} of the dissolved stain was measured in a multipurpose UV/VIS plate reader (VICTOR3 $_{TM}$; PerkinElmer).

5.2.5 Biofilm stability and inhibition assays

The biofilm stability against sodium metaperiodate (SM), proteinase K (PK) or DNase I treatment was tested as described previously (O'Neill et al. 2008; Rohde et al. 2005; Kaplan 2004). Briefly, 200 µl of an overnight grown culture diluted to an OD_{600} of 0.005 in BHI-glucose, were pipetted into sterile 96-well polystyrene microtiter plates and statically incubated overnight at 37°C. After 24 h incubation, the growth medium was replaced with 200 µl solution of SM (10 mM in 50 mM sodium acetate), of PK (Qiagen GmbH, 1 mg/ in 100 mM NaCl, 20 mM Tris/HCl, pH 7.5) or of DNase I (Sigma, 2 mg/ in 5 mM $MgCl_2$). Subsequently, plates were incubated at 37°C for 2 h and the remaining biofilms were quantified as explained above.

To assess the effect of specific anti-SesC antibodies (αSesC-IgGs) produced as earlier described (Shahrooei et al. 2009) on biofilm formation, $1x10^6$ bacteria were in the first instance incubated with αSesC-IgGs (20 µg/ bacterial suspension) for 2 h at 4°, and in a volume of 200 µl medium brought into a 96-well plate. Plates were incubated overnight at 37°C without shaking to allow bacterial growth and biofilm formation.

5.2.6 PIA quantification by PIA non-specific immunoblot assay

The relative amount of PIA present in a biofilm was determined as described (Singh et al. 2010), however with some modifications. Briefly, 1 of a diluted overnight culture of bacterial suspension ($5x10^6$ CFU/) in BHI-NaCl or BHI-glucose were pipetted in 24-well polystyrene microtiter plates (BD Biosciences) and next, plates were incubated overnight at 37°C. After incubation, spent medium was removed, 500 µl PBS was added into each well and the biofilm mass was removed from the surface via pipetting. Samples were transferred to 1.5 tubes which were next centrifuged for 3 min at 12000×*g*. Pellets and biofilm material were re-suspended in 0.5 M EDTA (pH 8) to an OD_{600} of 0.5 and PIA was extracted by boiling the samples for 5 min. After centrifugation at 18000×*g*, 250 µl of the supernatant was added to an Eppendorf tube with 25 µl PK solution (20 mg/). The mixture was incubated for 1 h at 60°C and afterwards PK was deactivated for 30 min at 80°C. Sample aliquots were applied to a nitrocellulose membrane, which was blocked with 5 % (w/v) bovine serum albumin (BSA) in TTBS [Tris-buffered saline (100 mM Tris/HCl, 0.9% NaCl) with 0.05 % Tween 20]. After washing the membrane 3 times in TTBS, it was incubated

overnight at 4°C with wheat germ agglutinin-horseradish peroxidase conjugate (EY laboratories) in 1% (w/v) BSA-TTBS. After washing the membrane 3 times in TTBS, the presence of PIA was detected by the addition of Western blotting detection reagent (Amersham™ ECL, GE Healthcare), and visualized with a ChemiDoc™ XRS+ System (Bio-Rad).

5.2.7 Detection of SesC

Western blot analysis of SesC was performed as described (Hu et al. 2011). Cells of 10 overnight bacterial cultures grown at 37°C in BHI-glucose were harvested by centrifugation, resuspended in lysis buffer [10 mM Tris/HCl, 10 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.5, 100 µg lysozyme/, 100 µg lysostaphin/] and incubated for 1 h at 37°C. Then, cells were broken by passing them three times through a French press at 69 MPa (SLM Aminco) followed by sonication (Branson 2510, 42 kHz) of samples on ice. Proteins were electrophoretically separated on a 12% sodium dodecyl sulphate polyacrylamide gel and subsequently transferred onto a PVDF membrane. The primary antibody (1:5000 dilution of rabbit anti-SesC polyclonal antibodies in TTBS) was added to the membranes, which were left overnight, followed by horseradish peroxidase-conjugated anti-rabbit IgGs as secondary antibodies [1:2500 dilution in TTBS+1% (w/v) skim milk] for 2 h. Finally, the presence of SesC was visualized using ECL Western blotting detection kit (GE Healthcare) in combination with a ChemiDoc™ XRS+ System (Bio-Rad).

5.2.8 Scanning Electron Microscopy

In vitro biofilm formation on cover Glasses (Ø 10 mm, Menzel GmbH) was visualized by scanning electron microscopy (SEM) as described (Pintens et al. 2008). Briefly, an overnight bacterial culture was diluted in BHI-glucose to an OD_{600} of 0.005, and 1 of the diluted culture was pipetted into the wells of sterile 24-well polystyrene microtiter plates, which each contained a glass disk. Plates were incubated overnight at 37°C without shaking, after which the disks were washed 3 times with PBS. Biofilms formed on the disks were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) by incubation for 2 h at room temperature. After fixation, disks were rinsed with 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min with three changes. Thereafter, a post-fixation step was done with 1% osmium tetroxide for 2 h at 4°C. Next, disks were rinsed with distilled water (2 times, 10 min) and then dehydrated in 10 min steps in a series of ascending ethanol baths (25%, 50%, 75%, 95% and 100%). Following a bath of hexamethyldisilazan, dehydrated air-dried

samples were mounted on support stubs with C-stickers and silver glue, and sputter coated with platinum (Agar Scientific, Auto Sputter Coater). Finally, the samples were observed and images taken with a JSM7401F field emission scanning electron microscope (JEOL) in a high vacuum mode with a conventional Everhart-Thornley detector at 5kV accelerating voltage.

5.3 Results

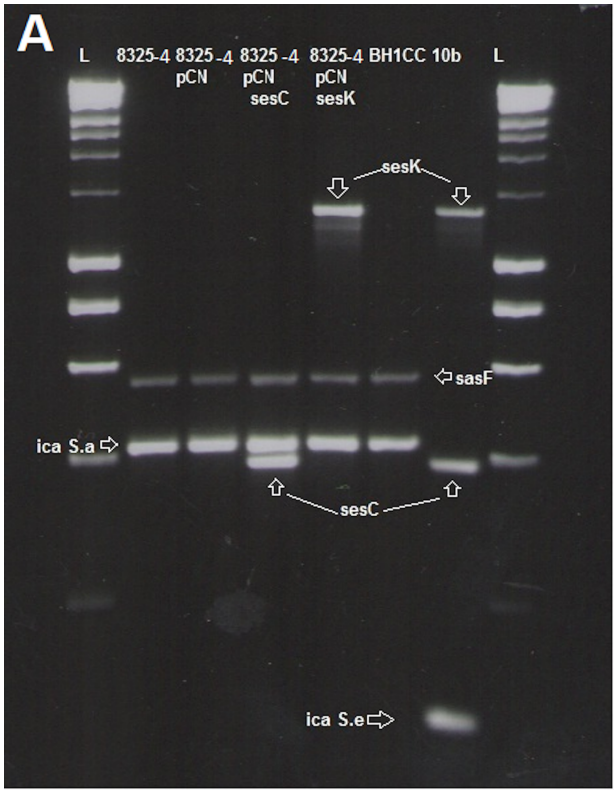
5.3.1 Heterologous expression of *sesC* in *S. aureus* switches the biofilm phenotype from PIA-dependent to proteinaceous

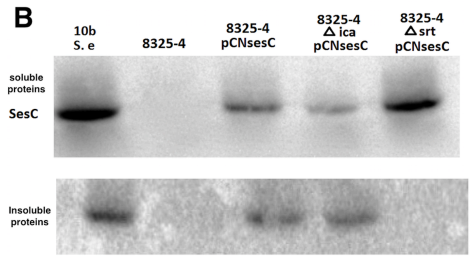
The *S. epidermidis sesC* gene was cloned into a pCN plasmid resulting in pCN*sesC*. The recombinant plasmid was introduced into the laboratory *S. aureus* strain 8325-4, which makes a PIA-type biofilm, and into the hospital-associated MRSA strain BH1CC, which makes a eDNA and proteinaceous biofilm (AtlE/FnBP-dependent) (Houston et al. 2011; Pozzi et al. 2012). The expression of *sesC* and the presence of the corresponding protein were confirmed by gel-based RT-PCR assay and Western blot assay (Figures 5.1A and B). Western blot confirmed the expression of SesC in the transformant strains. Heterologous expression of *sesC* had no effect on the BH1CC biofilm phenotype (data not shown), but inhibited biofilm formation by *S. aureus* 8325-4 transformants cultivated in BHI-NaCl (Figures 5.1C and D). Furthermore, 8325-4 (pCN*sesC*) biofilms grown in BHI-glucose were dispersed with proteinase K (PK) but not sodium metaperiodate (SM), which did disperse wild-type *S. aureus* 8325-4 biofilms (Figures 5.1C and D). This is consistent with a biofilm phenotypic switch from PNAG- to protein-mediated in these transformants. Nevertheless, quantification of PIA showed no changes in the rate of PIA production in *S. aureus* 8325-4 (pCN*sesC*) in comparison to the wild-type strain (Figure 5.1E).

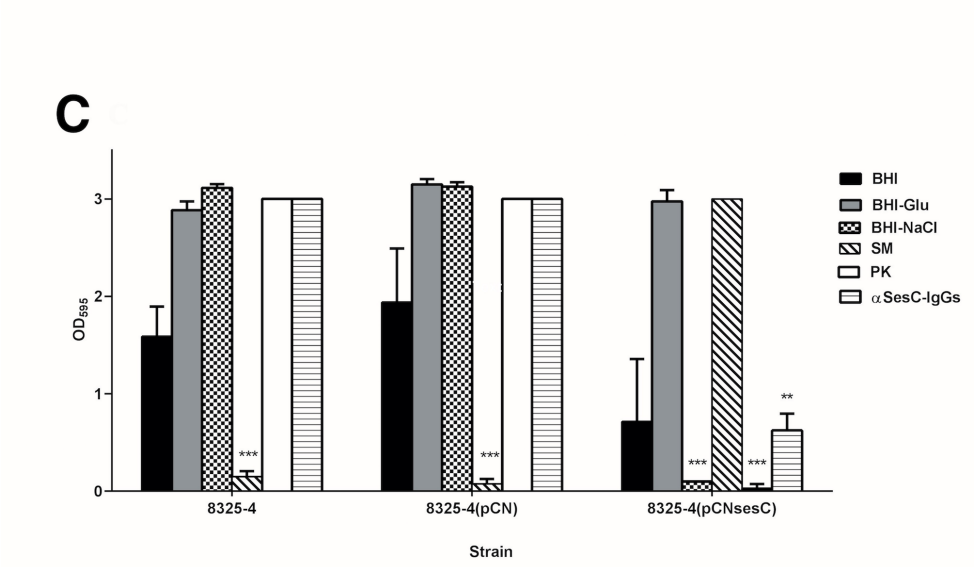
In order to confirm the relation between SesC production and a phenotypic switch of biofilm growth, another *ica*-positive, PIA-dependent biofilm-forming *S. aureus* strain, the clinical isolate MSSA4, was transformed with pCN*sesC*. As observed in 8325-4, the biofilm phenotype of MSSA4 switched from PIA-dependent to proteinaceous following introduction of pCN*sesC* (Figure 5.1F).

Moreover, the effect of αSesC-IgGs on biofilm formation by *S. aureus* strains 8325-4 and 8325-4 (pCN*sesC*) grown overnight in BHI-glucose was investigated. αSesC-IgGs had no effect on 8325-4 or 8325-4 carrying the empty plasmid, but inhibited biofilm formation by

8325-4 (pCN*sesC*) up to 80% (Figures 5.1C and D). Treatment with DNaseI did not have any significant impact on the biofilms (data not shown).







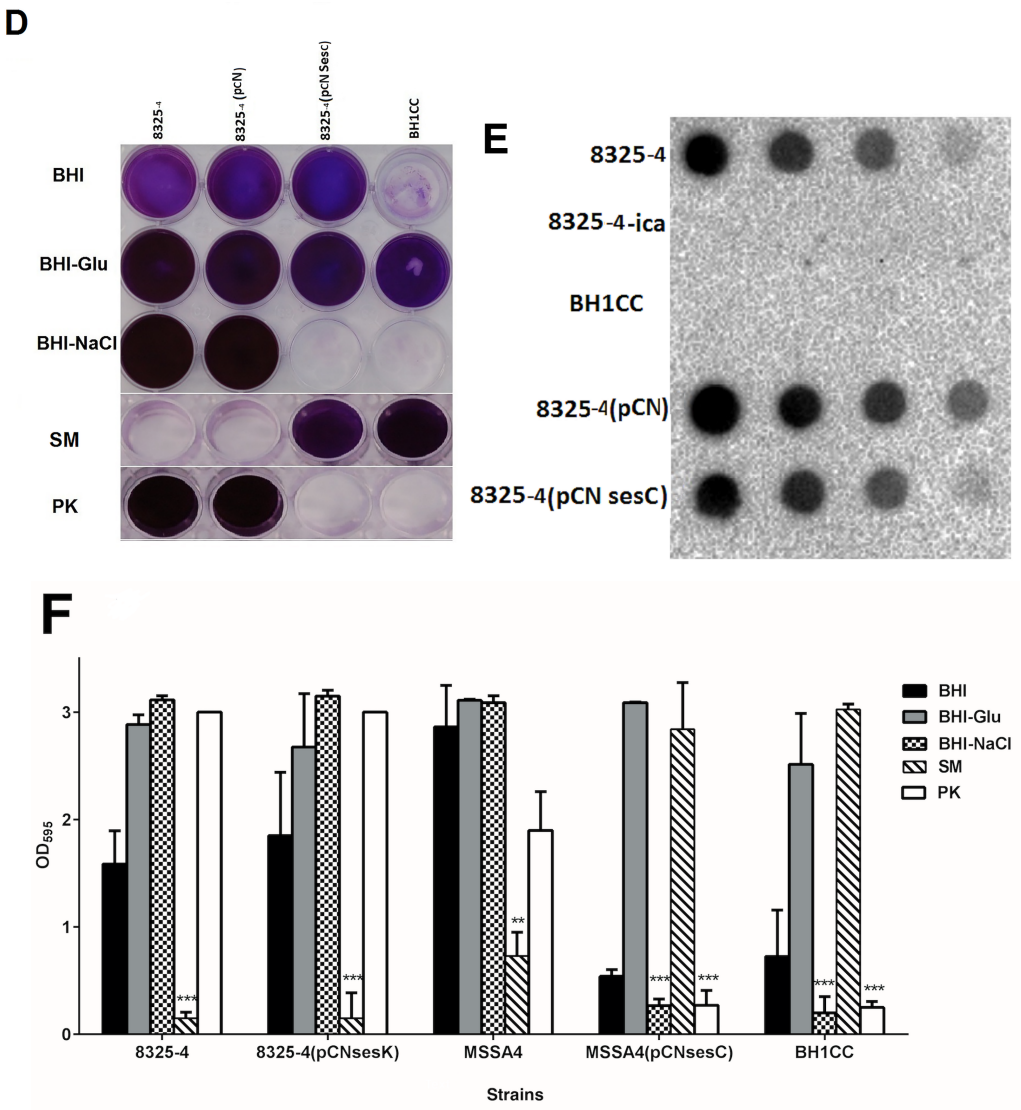


Figure 5.1. Effect of transformation of *S. aureus* strains with *sesC* or *sesK* on the biofilm formation. Using a semi-quantitative microtiter plate assay the level of biofilm formation in different media, the phenotype of biofilms and effect of αSesC-IgG antibodies on biofilm formation of different strains were identified. Biofilm formation in medium supplemented with NaCl or the effect of dispersal agents were used to discriminate the phenotype of biofilms. **(A**) Expression of *sesC*, *sesK*, *sasF* and *icaA* in cDNA of transformed strains was evaluated using the gel-based reverse transcription-PCR assay. **(B**) Detection of SesC production in *S. epidermidis* 10b, 8325-4, 8325-4 (pCN*sesC*) and 8325-4 *ica*::*tet* (pCNsesC) 8325-4 *srt*::*tet* (pCNsesC)) in soluble and insoluble fractions via Western blot assay. (**C**) The effect of *S. aureus* 8325-4 transformation with *sesC*. Introduction of a plasmid carrying *sesC* but not the mock plasmid changed the biofilm formation of transformants in BHI-NaCl and also the effects of PK and SM were opposite. (**D**) Microtiter plate assay demonstrating the effect of the inducers of biofilm formation (glucose and NaCl) and dispersal agents on biofilm formation of strains **(E)** quantification of PIA production using PIA non-specific immunoblot assay for biofilm in BHI-Glu. (**F**) Effect of transformation with *sesK* on the biofilm formation of strain 8325-4 and also the effect of *sesC* on the biofilm formation of strain MSSA4 in comparison with MRSA strain BH1CC. (SM: sodium metaperiodate, PK: proteinase K; error bars mean standard deviation)

Scanning electron microscopy (SEM) images from *S. aureus* 8325-4, 8325-4 with mock plasmid, 8325-4 (pCN*sesC*) and BH1CC biofilms, respectively, showed morphological differences (Figure 5.2). In BHI-glucose, *S. aureus* 8325-4 formed porous and less condensed biofilms whereas 8325-4 (pCNsesC) formed more condensed and smoother biofilms with a glue-like matrix as seen in BH1CC biofilms. *S. aureus* 8325-4 *ica::tet* did not form biofilm whereas 8325-4 *ica::tet* (pCNsesC) presented as a biofilm forming strain (Figure 5.2). To evaluate whether the phenomenon of biofilm phenotypic switching is due to the specific function of SesC or to high-level constitutive production of any LPxTG surface protein, the *sesC* gene in pCN*sesC* was replaced with *sesK* that encodes another LPxTG protein in *S. epidermidis*. Unlike s*esC*, which is present in all *S. epidermidis* strains, *sesK* is only present in circa 10% of *S. epidermidis* isolates (Shahrooei et al. 2012). Additionally, it was previously shown that anti-SesC antibodies could reduce *S. epidermidis* biofilm formation, whereas anti-SesK antibodies had no effect (Shahrooei et al. 2012). Transformation with pCN*sesK* had no impact on the biofilm phenotype of *S. aureus* 8325-4 (Figure 5.1F), showing that the effect of heterologous expression of *sesC* in PIA-producing *S. aureus* may be specific.

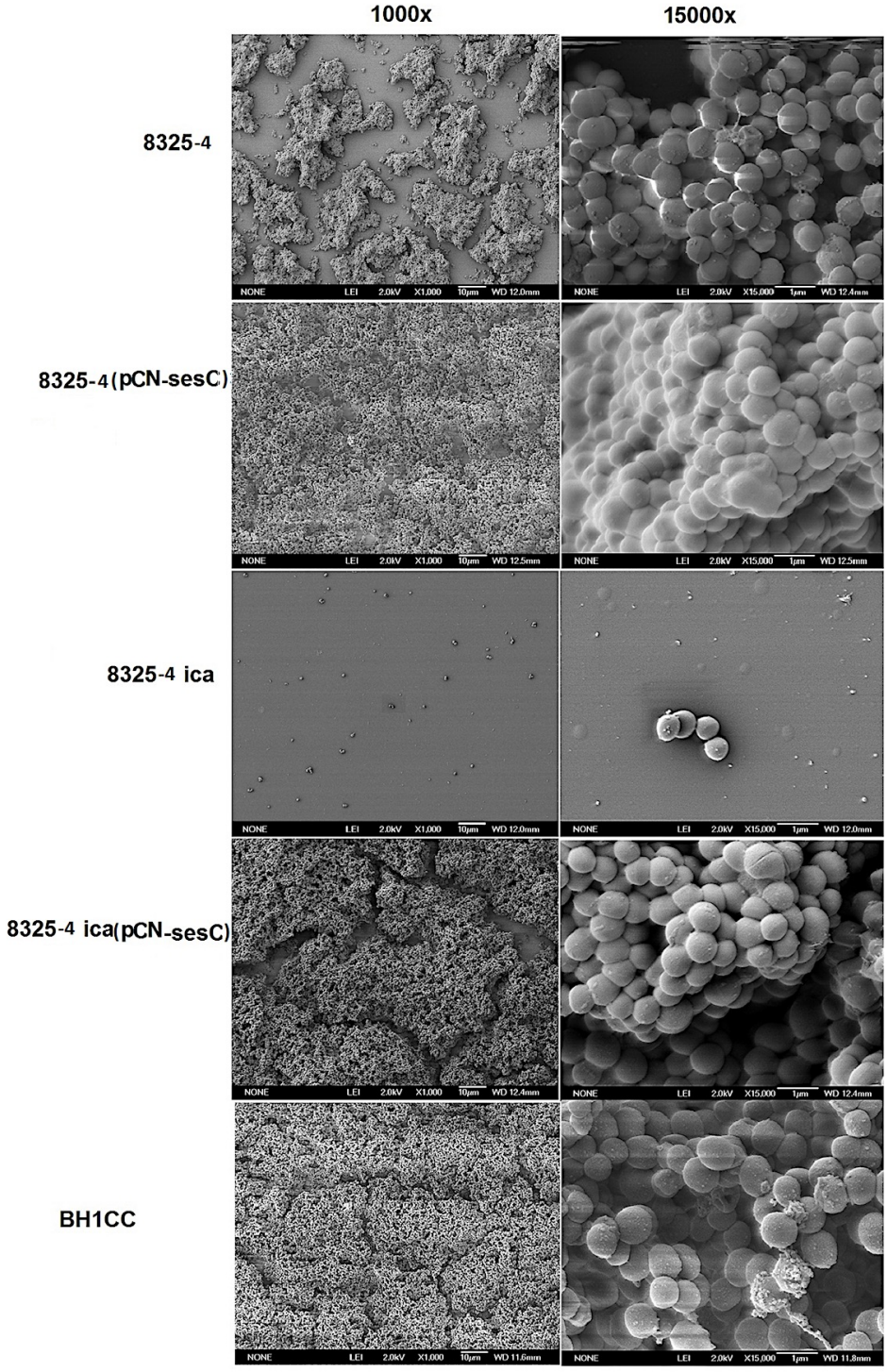


Figure 5.2. SEM images of biofilms formed by *S. aureus* 8325-4 and 8325-4 *ica*, their *sesC*-expressing transformants and BH1CC as controls. Biofilm growth on glass disks was allowed during overnight incubation at 37°C in BHI supplemented with 1% glucose. The next day, samples were fixed and sputter coated with platinum. The images show bacteria attached on the surface of disks at 1000x, 15000x magnification.

5.3.2 Expression of *sesC* promotes biofilm production by an *ica* mutant of *S. aureus* 8325-4

Previously published data revealed that deletion of the *ica* operon encoding PIA biosynthesis impaired PIA-dependent biofilm production by *S. aureus* 8325-4 but had no impact on the biofilm formation by MRSA strain BH1CC which expresses an AtlE/FnBP-mediated biofilm phenotype (Houston et al. 2011). Transformation of *S. aureus* 8325-4 *ica*::*tet* with *sesC* - in contrast to *sesK* - restored the biofilm formation to approximately wild-type levels in BHI-glucose (Figure 5.3). Furthermore, SEM analysis showed that the morphology of *S. aureus* 8325-4 *ica*::*tet* (pCN*sesC*) biofilms was similar to 8325-4 (pCN*sesC*) biofilm (Figure 5.2). Interestingly, when grown in medium supplemented with NaCl, which induces a PIA-type biofilm, *S. aureus* 8325-4 *ica*::*tet* (pCN*sesC*) was unable to produce biofilm while in BHI-glucose biofilm growth of 8325-4 *ica*::*tet* (pCN*sesC*) occurred. In addition, these biofilms were dispersed by PK, indicating a proteinaceous biofilm phenotype for this transformed mutant (Figure 5.3).

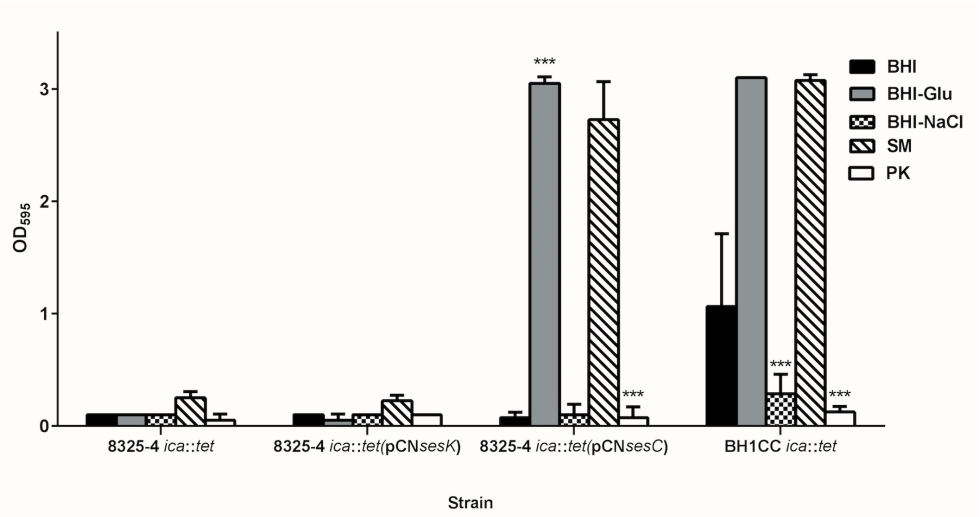


Figure 5.3. Effect of transformation with *sesC* and *sesK* on biofilm formation by non-biofilm-forming *S. aureus* 8325 *ica*::*tet* mutant. Transformation of this non-biofilm-forming mutant with *sesC* restored its biofilm formation and converted it to protein-mediated biofilm-forming strain that cannot form biofilm in the presence of NaCl and is dispersed by PK but not SM. Transformation with *sesK* had no effect. Mutation of the *ica* in PIA-independent biofilm-forming BH1CC strain had no effect on its biofilm formation.
(SM: sodium metaperiodate, PK: proteinase K; error bars mean standard deviation)

5.3.3 Surface expression of SesC is involved in biofilm formation

LPxTG proteins are known to be anchored to the bacterial peptidoglycan by sortases (Marraffini et al. 2006; Dramsi et al. 2005; Dramsi et al. 2008). Deletion of *srtA* inhibits

LPxTG protein-dependent biofilm formation. In 2008, O'Neill *et al.* reported that deletion of *srtA* in BH1CC impairs its biofilm forming activity, while biofilm formation of *S. aureus* 8325-4 *srtA*::*tet* was not affected (O'Neill et al. 2008). Introduction of pCN*sesC* in the latter strain, however, completely impaired biofilm production, further indicating the dominant role of SesC over PIA-type biofilm production (Figure 5.4). Unlike 8325-4, in the absence of sortase the *S. aureus* 8325-4 *srtA*::*tet* (pCN*sesC*) strain was unable to form biofilm. The presence of SesC in the soluble and insoluble proteins fractions of the transformant strain was investigated and showed that in the *S. aureus* 8325-4 *srtA*::*tet* (pCN*sesC*) SesC is only expressed in the soluble fraction (Figure 5.1B). This strongly suggests that presence of *srt* is necessary for sorting SesC to the cell wall.

Complementation of *S. aureus* 8325-4 *srtA*:: *tet* (pCN*sesC*) with the plasmid pSR*srtA*5 carrying the *S. aureus srtA* gene (O'Neill et al. 2008) restored biofilm formation (Figure 5.4). The biofilm of this complemented strain was only induced in BHI-glucose 1%, not in BHI-NaCl, and dispersed only with Proteinase K and not with sodium metaperiodate.

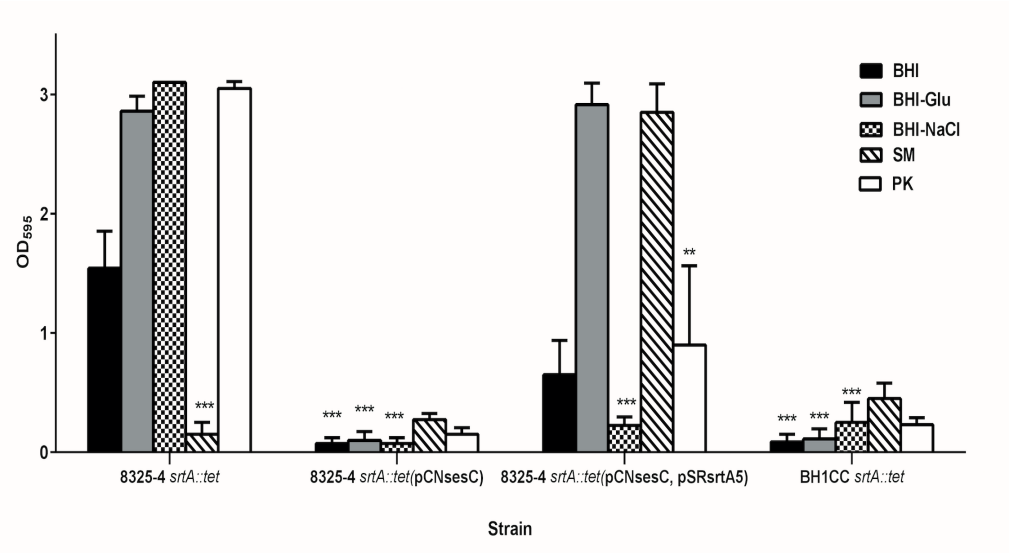


Figure 5.4. Effect of transformation with *sesC* on biofilm formation by the biofilm-forming isogenic *srtA* mutants of 8325-4. Effect of *srtA* mutation on biofilm formation of PIA-dependent biofilm-forming *S. aureus* 8325-4, (Atl/FnBP)-mediated biofilm-forming strain BH1CC, the *sesC*-tranformed 8325-4 *srtA*::*tet* strain and complementation of *S. aureus* 8325-4 *srtA*::*tet* (pCN*sesC*) with *srtA*, was evaluated using the quantitative microtiter plate assay. (SM: sodium metaperiodate, PK: proteinase K; error bars mean standard deviation)

5.4 Discussion

Surface proteins have been shown to play important roles in *S. epidermidis* and *S. aureus* biofilm formation, especially by MRSE and MRSA in device-related infections (Hennig et al. 2007; Houston et al. 2011; Mack et al. 2007). We previously reported that rabbit polyclonal antibodies directed against the extracellular domain of *S. epidermidis* LPxTG surface protein SesC (or αSesC-IgGs) can significantly inhibit *S. epidermidis* biofilm formation *in vitro* and *in vivo* in a rat model of subcutaneous CRI as well as in a mouse model of jugular vein CRI (Shahrooei et al. 2012). It has also been demonstrated that active immunization with the recombinantly produced extracellular domain of SesC decreased *S. epidermidis* biofilm formation in a rat model of subcutaneous CRI (Shahrooei et al. 2012). Data obtained in this study are consistent with previous observations and demonstrate that SesC plays an important role in biofilm formation even in another genetic background.

We have tried, but were unable to knock out *sesC*. It may be that knockout of *sesC* is associated with a lethal phenotype in these bacteria. On the other hand, using antisense RNAs to knock down *sesC* in *S. epidermidis* and subsequently applying gel-based reverse transcription-PCR (RT-PCR) assay and Western blot analysis, we could not see any changes in *sesC* expression in the transformed strain compared with the parental strain.

PCR screening to determine the incidence of *sesC* in 300 clinical isolates of *Staphylococcus* spp. including 175 *S. epidermidis*, 33 isolates of methicillin-resistant *S. aureus* (MRSA), 50 isolates of methicillin-sensitive *S. aureus* (MSSA) and 42 strains belonging to various non-*epidermidis* CoNS species, revealed that *sesC* is present in all *S. epidermidis* strains, but not in other staphylococci, indicating that it is potentially a conserved, *S. epidermidis*-specific gene (Khodaparast et al. 2016a).

Despite the lack of *S. epidermidis sesC* mutants, the unraveling of the function of SesC in biofilm formation was attempted by introduction of *sesC* into *S. aureus* strains that formed different types of biofilm (PIA-dependent, PIA-independent or non-biofilm-forming). Using gel-based RT-PCR assay and Western blot analysis, we confirmed *sesC* expression and the production of the corresponding protein in these transformed strains. Our findings revealed that transformation with *sesC* had no impact on the strains, which display proteinaceous biofilm phenotypes, while it caused a switch from PIA-dependent biofilm-formation to a proteinaceous-type biofilm in MSSA strains 8325-4 and MSSA4. As this biofilm phenotype switching was not associated with transformation of *S. aureus* 8325-4 with *sesK*, it can be concluded that the switch is not necessarily associated with high expression level of any

LPxTG surface protein or with the genetic background of the transformed strain, but is specifically caused by SesC. Results obtained also show that presence of SesC is dominant over the presence of PIA and impairs the role of PIA in cell-cell interaction.

To further confirm the direct involvement of SesC in biofilm formation, we transformed the non-biofilm-forming, isogenic *ica* mutant of *S. aureus* 8325-4 (being 8325-4 *ica*::*tet*) with *sesC*. Transformation with *sesC* converted this non-biofilm-forming mutant to a proteinaceous biofilm-forming strain similar to its parental strain transformed with *sesC*. These data suggest a direct role for SesC in biofilm formation, because even in the absence of PIA we have the same effect of SesC on biofilm formation.

To answer the question whether SesC affects PIA biofilm formation in mutated *srtA* strain, *S. aureus* 8325-4 *srtA*::*tet* was transformed with *sesC*. Deletion of *srtA* has no impact on biofilm formation of PIA-dependent biofilm-forming strain 8325-4, but impairs the normal display of LPxTG surface proteins, including SesC. After the introduction of *sesC*, we observed that this biofilm-forming strain converted to a non-biofilm-forming strain. To confirm the localization of SesC in 8325-4 *srtA::tet* (pCNsesC) strain, we checked the presence of SesC in the soluble and insoluble protein fraction. Since SesC is a cell wall-anchored LPxTG protein, basically we expected to find it in the insoluble fraction. However, SesC was observed only in the soluble part. These data suggest that the presence of sortase is necessary to sort SesC to the cell wall (Seddon et al. 2004).

Complementation of the non-biofilm-forming *sesC*-expressing mutant with *srtA* by means of transformation with pSRsrtA5 converted it to a proteinaceous biofilm-forming strain. These data confirm that transformation with *sesC* is sufficient to switch the mechanism of biofilm formation to a proteinaceous type biofilm on condition that SesC is sorted to its place on the surface, attached to the peptidoglycan layer. Our data show that SesC expression does not have any negative effect on PIA production. There is some evidence that may explain this phenomenon. Previous groups reported that the generation of PIA is not sufficient to form biofilm (Vergara-Irigaray et al. 2009). Vergara-Irigaray *et al.* showed that the clinical MRSA strain 132 is able to alternate between a proteinaceous and a polysaccharidic biofilm matrix, depending on environmental conditions, and strain S115 generates PIA but is a non-biofilm-forming strain. This might be because of the existence of a defect in the export of PIA by IcaC or IcaB (Vergara-Irigaray et al. 2009). Similarly to the effect of FnAB on biofilm phenotype in *S. aureus* strain S132 and BH1CC, there is the possibility that here, the extracellular location of SesC changes the architecture of the cell wall to the extent that PIA can no longer link the cells. This hypothesis is consistent with the

observed effect of biofilm dispersal agents on *sesC*- and *ica*-positive strains. The observation that high level expression of another surface protein (SesK) did not have a similar effect may suggest some form of interaction between SesC and PIA that is absent for SesK. Different expression levels of SesC and SesK in spite of using the same expression vectors may offer another explanation.

Expression of SasG, a surface protein in *S. aureus*, can similarly as SesC switch the biofilm of PIA-dependent *S. aureus* strains SH1000 and 8325-4 to protein-mediated biofilm (Corrigan et al. 2007) SasG like SesC is a fibrinogen-binding protein, and SasG masked binding to fibrinogen mediated by both ClfB and the FnBPs. Biofilm formation by SasG is also likely to be protease dependent, because the broad spectrum protease inhibitor a2-macroglobulin inhibited the biofilm formation process of the strain SH1000 transformed with *sasG* (Corrigan et al. 2007). By looking at the protein sequences alignment of SesC and SasG, we realized they have 26% identity.

Similar to the presented observations, a recent report illustrated the impact of introducing the methicillin resistance gene *mecA* into the PNAG-producing MSSA strain 8325-4 (Pozzi et al. 2012). This generated a heterogeneously oxacillin resistant (HeR) strain, from which a homogeneous, high-level resistant (HoR) derivative was isolated following exposure to oxacillin. Transcription of *icaADBC* and production of PNAG were impaired in the 8325-4 HoR derivative, which instead produced a proteinaceous biofilm that was significantly inhibited by antibodies against the *mecA*-encoded penicillin binding protein 2a (PBP2a). HoR derivatives of *S. aureus* 8325-4 *icaADBC*::*tet*, 8325-4 *fnbAB*::*tet*, 8325-4 *atl*::*cat* and 8325-4 *srtA*::*tet* exhibited a similar biofilm phenotype as 8325-4 HoR (Pozzi et al. 2012; McCarthy et al. 2015).

SEM images confirm the presence of a morphologically different biofilm for *S. aureus* 8325-4 in comparison to its transformant harboring pCN*sesC*. These findings are also supported by the effect of αSesC-IgGs on established biofilms of SesC-producing transformants.

The obtained *in vivo* data (chapter 6) are consistent with our previous findings in suggesting an important role for SesC in infection. Transformation with *sesC* increased the organ infection rate up to 100-fold. This can be partially explained by the fact that fibrinogen is one of the components of the extracellular matrix (Pereira et al. 2002). Our previous report showed that transformation of *S. aureus* strain RN4220 with *sesC* increased the fibrinogen-binding ability of transformants, suggesting SesC as a potential Fg-binding MSCRAMM (Shahrooei et al. 2009). But, our experiments also show enhanced adherence of the

transformant strains to an uncoated catheter *in vitro*, suggesting that the adherence effects are not entirely or solely mediated by binding to host factors.

CHAPTER VI: Development of a new Mouse Model to Study Staphylococcal Catheter-Associated Biofilm Infection

6.1 Introduction

S. epidermidis is a ubiquitous commensal and a part of the normal human flora, but they can easily attach to implanted devices and form biofilm (Ziebuhr et al. 2006). Medical device-related infections are typified by high morbidity, and often require device removal (Hanke & Kielian 2012).

Thus far, a subcutaneous rat model for CRIs was used (Shahrooei et al. 2009). Despite resembling intra-operative contamination with skin flora, this model does not mimic conditions found in the human intravascular system and has some limitations. The immune response at the site of infection in the subcutaneous model may not reflect the response to the intravascular device-related infections in the peripheral blood. Hence, this study was designed to develop an animal model for central venous catheter infections. This new model will be used in the future to evaluate the potential of vaccines and immunotherapy directed against *S. epidermidis* infections. This mouse model closely mimics the clinical situation of human infection (Parienti et al. 2015; Chauhan et al. 2016).

6.2 Materials and Methods

6.2.1 Jugular vein catheterized (JVC) mouse model

Materials used during the surgery are listed in Figure 6.1 (Shahrooei et al. 2012; Khodaparast et al. 2016b). Briefly, 4-weeks-old female Swiss-Webster mice (Taconic) were anesthetized with a single intraperitoneal (i.p.) injection of sodium pentobarbital (Nembutal, 40-60mg/kg body wt) and placed on a heating pad to maintain the body temperature at 37°C. An anesthetized and surgically prepared animal was then placed in dorsal recumbence under a dissecting microscope (Zeiss, Jena, Germany, 10x magnification). A small vertical incision was made using small scissors and the right jugular vein was identified, mobilized and exposed with blunt surgical dissection. A single lumen polyethylene catheter (internal ⦸ 0.011 inch (0.28 mm); outer ⦸ 0.024 inch (0.61 mm); insertion length, 1 cm; Intramedic, Becton Dickinson and Company, cat. no. 427400) was inserted into the right jugular vein and advanced into the superior vena cava via a small incision in the vein made with vein scissors (Figure 6.2). A ligature was then tied loosely around the catheter and patency was verified. Once blood flow had been established, the catheter was anchored in place. Subsequently, a small midline skin incision was made between the scapulae. The catheter was subcutaneously tunneled by a straight surgical clamp and exteriorized through midline scapular incision. The incisions were then closed with stitches. The patency was tested and

the catheter was flushed with 100 µl of saline, sealed with a plug and left in place throughout the experiment. Thereafter, mice were housed separately and monitored for recovery (Figure 6.2). 24 h after surgery, the mice were inoculated via the catheter lumen with 100 µl of an *S. aureus* suspension (OD_{600}=0.03, containing ≈3x10^7 CFU). Then the catheter was flushed again with 100 µl saline so that bacteria entered the venous system of animals.

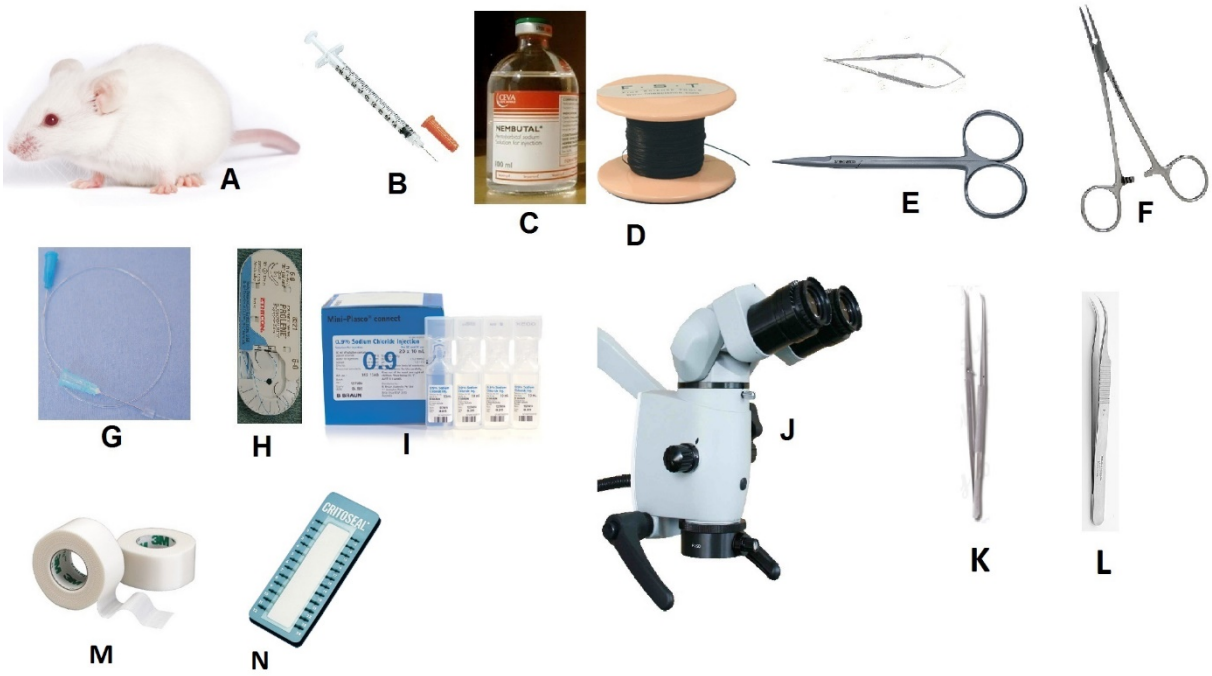


Figure 6.1 Materials for surgery. A. Swiss mouse **B.** insulin syringe **C.** Nembutal **D.** Suture Threat **E.** small surgical scissors **F.** 2 Halstead Surgical Clamp **G.** small Mouse Catheter **H.** Sterile Surgical Silk Suture **I.** 0.9 NaCl solution **J.** surgical microscope **K.** Microsurgery-Forceps-Small **L.** Curved-tipped microsurgery forceps, **M.** surgical tape **N.** Critoseal

After taking the blood samples at day 1 or 5 post-infection, animals were euthanized by CO_2 inhalation and catheters and organs (spleen, liver, heart, vein and right kidney) were aseptically harvested from euthanized animals. The harvested organs were mechanically homogenized in saline. The portion (~1 cm) of catheter that was inside the vein was cut gently, washed and placed in a tube containing 1 saline. Tubes containing the catheter fragments were vortexed for 10 s, sonicated for 5 min at 40 kHz in a water bath (Branson 2200, Branson Ultrasonics) and again vortexed for 10 s. Serial dilutions of the organ homogenates and catheter fluid collections were cultured on blood agar plates using a spiral plating system, and plates were incubated at 37°C overnight. Colonies on all plates were counted and the number of bacteria was defined as the mean of at least 5 quantitative cultures. All *in vivo* experiments were repeated at least twice and conducted in compliance

with the guidelines for animal experimentation. The Institutional Animal Care Commission and Ethical Committee of the KU Leuven approved all experimental protocols.

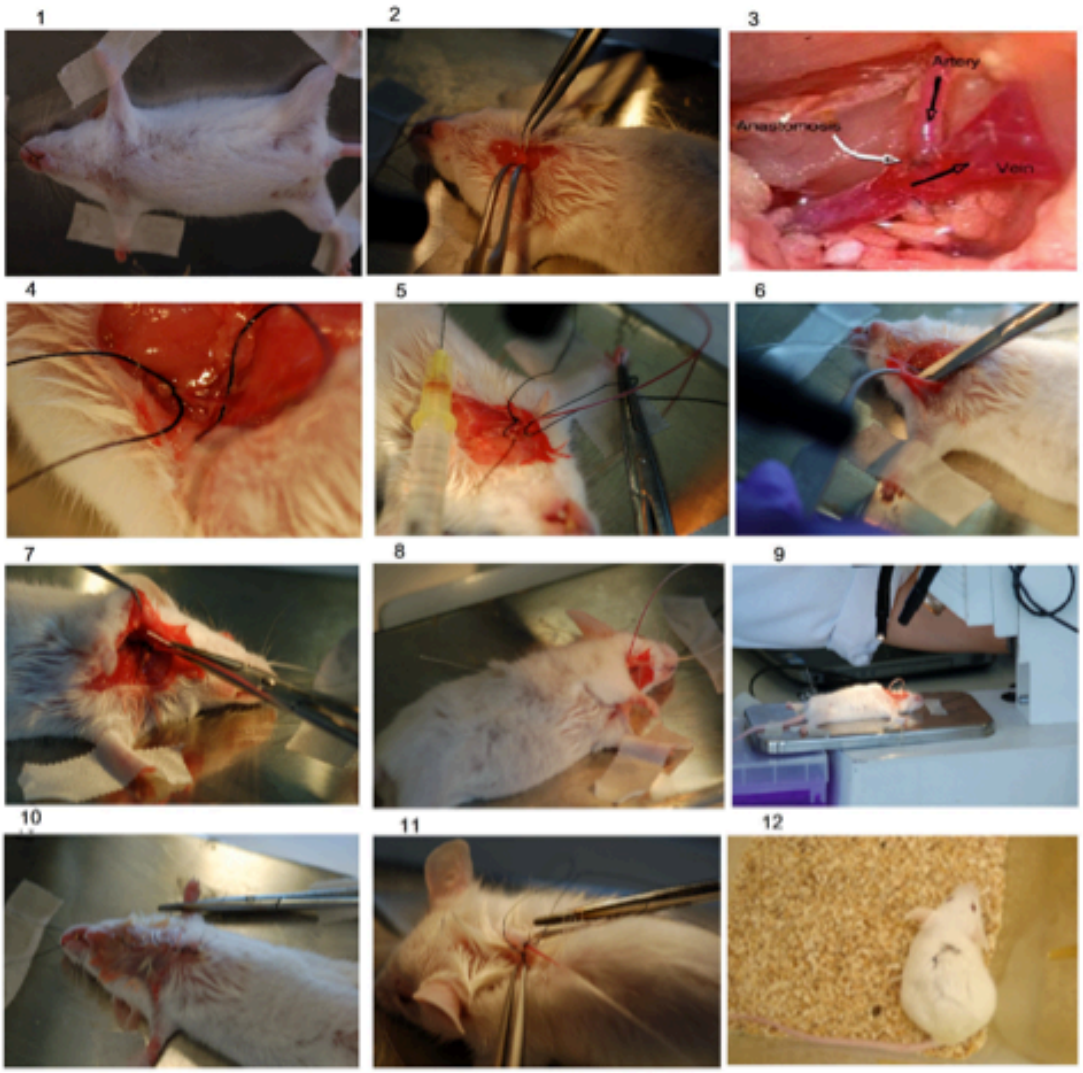


Figure 6.2. Jugular vein catheterization in mice **1.** An anesthetized and surgically prepared animal was placed in dorsal recumbence under a dissecting microscope, **2.** A small vertical incision was made using small scissors and the right jugular vein was identified, **3&4.** A single lumen polyethylene catheter, 1 cm, was inserted into the right jugular vein and advanced into the superior vena cava via a small incision in the vein made by vein scissors, **5.** Once blood flow had been established, the catheter was anchored in place and the patency was verified, **6.** A small midline skin incision was made between the scapulae, **7& 8.** The catheter was subcutaneously tunneled by a straight surgical clamp and exteriorized through midline scapular incision. **9, 10& 11.** The incisions were closed with stitches, **12.** 24 h after surgery.

6.2.2 Investigation of the effect of SesC on the *in vitro* attachment to a catheter surface

In vitro bacterial attachment to the surface of a commercial polyurethane (PU) intravenous catheter (Arrow International) was examined as described (Pintens et al. 2008) with some modifications. Overnight cultures of *S. aureus* strains 8325-4 and 8325-4 (pCN*sesC*) were washed with saline (0.9% NaCl) and diluted to an OD_{600} of 0.03 in saline. Seven mm catheter fragments were added to 2 of bacterial suspension and the mixture was incubated at 37°C. After 2 h incubation, catheters were removed. After gentle rinsing with saline, catheters were placed in a tube containing 1 saline. Tubes were vortexed for 10 s, sonicated for 10 min at 40 kHz using a Branson water bath and again vortexed for 10 s. Thereafter, tube contents were 10-fold serially diluted and 50 µl aliquots of each dilution were plated on TSA plates using a spiral plater system (Spiral Plater Systems, Inc. Cincinnati, Ohio), and plates were incubated at 37°C overnight. Colonies were counted and the number of bacteria was defined as the mean of at least five quantitative cultures.

6.2.3 The effect of rabbit polyclonal αSesC-IgGs on *S. epidermidis* infection in the JVC model

For this experiment, an overnight culture of *S. epidermidis* strain 10b, grown to the late exponential/stationary growth phase in BHI, was pelleted, resuspended, and diluted to an OD_{600} of 0.3 (~$3x10^8$) in 0.9% NaCl. Three inoculums were taken, one without any IgG, while two others were mixed with either preimmune or αSesC-IgGs (80 µg/) and incubated for 2 h at 4°C. After a 24-h recovery from surgery, 150 µl (~$5x10^7$ CFU) of the mixture was injected through the catheter lumen. Animals in the first, second and third groups received bacteria, bacteria preincubated with preimmune IgGs, and bacteria preincubated with αSesC-IgGs, respectively. After the blood samples were obtained at day 5 post-infection, the animals were sacrificed. The catheters were aseptically removed, and the portion (1 cm) that was inside the vein was cut, gently washed, and processed for quantitative culturing as explained above. To compare the overall infection rate, the spleens, livers, hearts, veins, and right kidneys were aseptically harvested, mechanically homogenized in 0.9% NaCl, and processed for quantitative cultures as explained above.

6.2.4 The study of the involvement of *sesC* in virulence and biofilm formation

Nine mice were divided into 3 groups of 3 mice. Overnight cultures of *S. aureus* strains 8325-4, 8325-4 (pCN68) and 8325-4 (pCN68sesC) grown to the late exponential/ stationary growth phase in (selective) BHI medium, were pelleted, re-suspended and diluted to an OD_{600} of 0.03 in 0.9% NaCl with the suitable antibiotics. After a 24 h recovery period, animals in one group were inoculated through the catheter lumen with 100 µl of one of 3 the bacterial suspensions (~$3x10^7$ CFU) again with suitable antibiotics. Five days post-infection, the infection rate and the bacterial load on the implanted catheter were determined and compared between the 3 groups.

6.3 Results

6.3.1 The effect of αSesC-IgGs on the overall infection rate by *S. epidermidis*

To investigate the effect of αSesC-IgGs antibodies *in vivo*, the mean numbers of bacteria recovered from the blood, catheters, veins, livers, spleens, hearts, and kidneys of animals in different groups were compared. Pre-incubation of bacteria with αSesC-IgGs significantly reduced the number of bacteria recovered from the catheter, vein, spleen, heart, liver, and kidney by 26-, 71.5-, 331-, 327-, 215-, and 52-fold, respectively ($P<0.01$, one-way ANOVA), whereas pre-incubation of bacteria with pre-immune IgGs had no effect on the infection rates (Figure 6.3). Almost no bacteria were found in the circulation (Shahrooei et al. 2012).

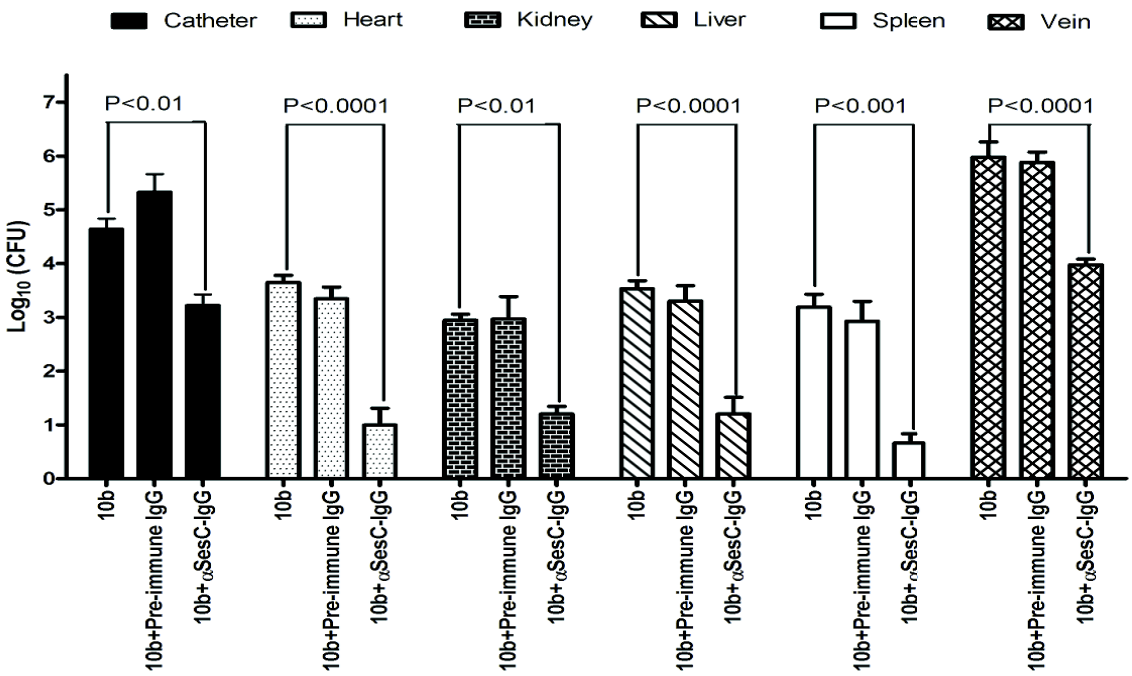


Figure 6.3. Effect of pre-incubation of *S. epidermidis* strain 10b with pre-immune or αSesC-IgGs on the overall infection rates in the JVC mouse model. A single lumen polyethylene catheter was surgically inserted into the right jugular vein of Swiss-Webster mice. After 24 h, an overnight culture of *S. epidermidis* strain 10b (~$3x10^8$+) in 0.9% NaCl was mixed with pre-immune or αSesC-IgGs (80 μg/) or the same volume of PBS, followed by incubation for 2 h at 4°C. After a 24-h recovery from surgery, 150 μl(~$5x10^7$) of the mixture was injected through the catheter lumen. After 5 days, the overall infection rates in different organs and the catheter colonization were quantified. Data for each group were obtained from bacteria recovered from the catheter or organs of six infected mice (three mice per group in each experiment, three groups, two independent experiments). The error bars indicate the standard errors of the mean (Shahrooei et al. 2012).

6.3.2 Heterologous expression of *sesC* increases colonization of polyurethane intravenous catheters *in vitro* and *in vivo*

Transformation of *S. aureus* 8325-4 with *sesC* significantly increased its attachment to polyurethane intravenous catheters *in vitro* (P<0.01; 1-way ANOVA) (Figure 6.4 A). Using a jugular vein catheterized (JVC) mouse model, the number of bacteria recovered from the catheter implanted in animals and afterwards infected with *S. aureus* 8325-4 (pCN*sesC*) was also significantly higher than after administration of *S. aureus* 8325-4 (P<0.05; 1-way ANOVA) (Figure 6.4 B). Interestingly, 8325-4 (pCN*sesC*) did not only show an increased catheter colonization ability compared to the non-transformed strain, but also the overall infection rate was raised. The number of 8325-4 *versus* 8325-4 (pCN*sesC*) cells in blood were similar, but the number of *S. aureus* 8325-4 (pCN*sesC*) cells recovered from organs such as spleen, liver, heart, vein and kidney were significantly increased compared to *S. aureus* 8325-4 (10- to 100-fold, P<0.05; 1-way ANOVA) (Figure 6.4 B). These results

indicate that SesC is a colonization factor that may promote *S. epidermidis* catheter and organ colonization, which in turn is the first step in biofilm formation and the establishment of a chronic infection.

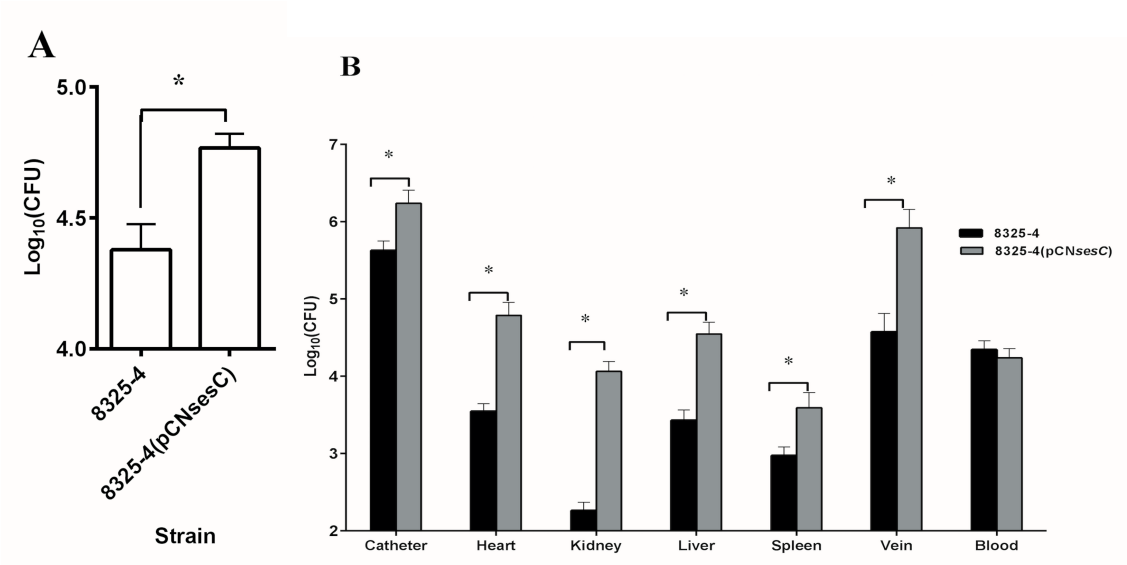


Figure 6.4. Effect of transformation with *sesC* on *in vitro* and *in vivo* catheter colonization and *in vivo* organ colonization. (**A**) Seven mm catheter fragments were inoculated with *S. aureus* 8325-4 or its *sesC*-expressing transformant. After 2 h incubation at 37°C, catheters were rinsed and adherent bacteria were detached by sonication and the numbers of bacteria recovered from each catheter fragment were quantified by quantitative cultures. (**B**) *In vivo*, catheterized animals were inoculated through the catheter lumen with *S. aureus* 8325-4 strain or its 8325-4 (pCN*sesC*) transformant. At day 5 post-infection, the numbers of bacteria attached to the catheters or recovered from organs were quantified by quantitative cultures. The *sesC*-expressing transformant 8325-4, (pCN*sesC*) has a higher rate of colonization of different organs up to 100-fold compared to its parental strain. *: $P<0.05$

6.3.3 Antibodies against SesC have a therapeutic effect in a *S. aureus* strain 8325-4 (pCN*sesC*)-induced catheter-related infection

The rate of catheter and organ colonization significantly decreased (100-100.000 fold; $P<0.01$-0.001; 1-way ANOVA) in the JVC mouse model group inoculated with 8325-4 (pCN*sesC*), which was pre-incubated with αSesC-IgGs *versus* untreated 8325-4 (pCN*sesC*) (Figure 6.5). Pre-immune IgGs had no significant effect on the catheter and organ colonization by *S. aureus* 8325-4 or its *sesC*-expressing transformant (data not shown).

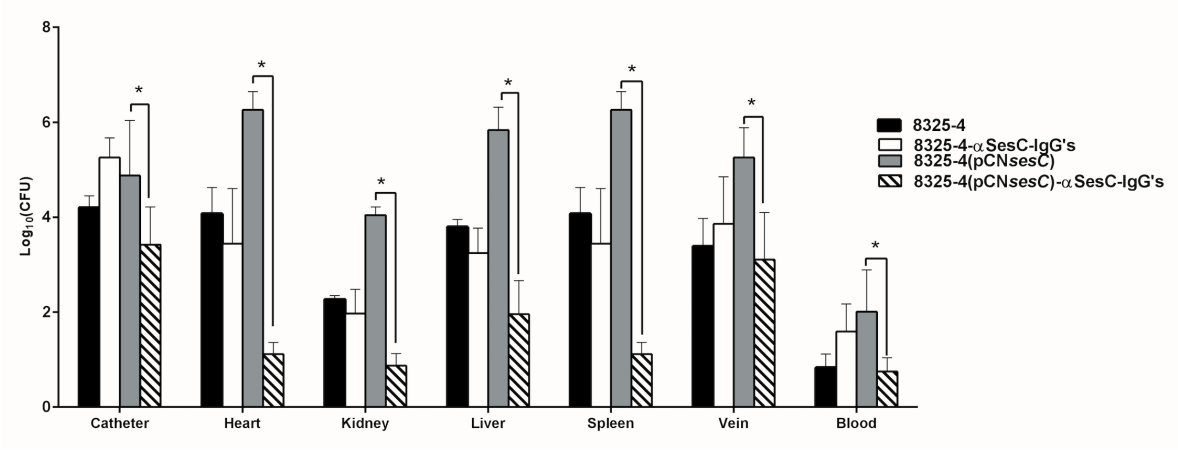


Figure 6.5 Effect of αSesC-IgGs on catheter colonization and infection rate by 8325-4 and its *sesC*-expressing transformant. Catheterized animals were inoculated with bacteria pre-incubated with αSesC-IgGs or pre-immune IgGs for 2 h at 4°C. The next day, animals were sacrificed and the bacteria were recovered from the catheter and organs and quantified by quantitative cultures. Pre-incubation with αSesC-IgGs significantly reduced the rate of catheter and organ colonization by 8325-4 (pCN*sesC*) from 10 to 10000-fold. *p<0.05

6.4 Discussion and Conclusion

The immune response at the site of infection in the subcutaneous rat model that we have used so far may not completely reflect the immune response to the intravascular device-related infections in peripheral blood. In this study, we developed and used a mouse JVC model to investigate the effect of anti-*sesC* antibodies. The transformant strains with *sesC* showed a significantly better attachment to the catheter in comparison to the parental strains. This higher colonization suggests a role for SesC in attachment to the catheter surfaces.

Reduction of *in vivo* catheter and organ colonization by SesC-producing *S. aureus* strains in the presence of αSesC-IgGs indicate the specificity of the antibody, surface expression of SesC, and involvement of SesC in catheter and organ colonization (Khodaparast et al. 2016b).

Although, we have to be cautious in extrapolating conclusions based on data obtained in *S. aureus* to *S. epidermidis*, we conclude that SesC is a virulence factor associated with the early stages in *S. epidermidis* biofilm formation, such as adhesion and colonization. This way SesC may favor chronic, persistent infections on indwelling biomaterials. The biofilm formation abilities of *S. epidermidis* may be due in part to the presence of SesC and similar factors (Khodaparast et al. 2016b).

CHAPTER VII: Opsonophagocytosis of planktonic and sessile bacteria by αSesC-IgGs

Vaccination with SesC Decreases *Staphylococcus epidermidis* Biofilm Formation

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The increased use of medical implants has resulted in a concomitant rise in device-related infections. The majority of these infections are caused by *Staphylococcus epidermidis* biofilms. Immunoprophylaxis and immunotherapy targeting *in vivo*-expressed, biofilm-associated, bacterial cell surface-exposed proteins are promising new approaches to prevent and treat biofilm-related infections, respectively. Using an *in silico* procedure, we identified 64 proteins that are predicted to be *S. epidermidis* surface exposed (Ses), of which 36 were annotated as (conserved) hypothetical. Of these 36 proteins, 5 proteins—3 LPXTG motif-containing proteins (SesL, SesB, and SesC) and 2 of the largest ABC transporters (SesK and SesM)—were selected for evaluation as vaccine candidates. This choice was based on protein size, number of antigenic determinants, or the established role in *S. epidermidis* biofilm formation of the protein family to which the candidate protein belongs. Anti-SesC antibodies exhibited the greatest inhibitory effect on *S. epidermidis* biofilm formation *in vitro* and on colonization and infection in a mouse jugular vein catheter infection model that includes biofilms and organ infections. Active vaccination with a recombinant truncated SesC inhibited *S. epidermidis* biofilm formation in a rat model of subcutaneous foreign body infection. Antibodies to SesC were shown to be opsonic by an *in vitro* opsonophagocytosis assay. We conclude that SesC is a promising target for antibody mediated strategies against *S. epidermidis* biofilm formation.

The experiments described in the following chapter were carried out by L. Khodaparast and published as a part of the paper of Shahrooei et al. 2012 (Infect Immun. 2012 Oct;80(10):3660-8. doi: 10.1128/IAI.00104-12. Epub 2012 Jul 16).

7.1 Introduction

The OpsonoPhagocytic Killing (OPK) assay is one of the assays used to measure the functional capacities of vaccine-candidate-raised antibodies. This *in vitro* assay can help to demonstrate if the vaccine-induced antibodies drive efficient complement deposition and subsequent opsonophagocytic killing (Boackle 1993). It has been already shown that among five potential surface-exposed "Ses" proteins of *S. epidermidis*, SesC is the most immunogenic protein (Shahrooei et al. 2012). To understand the immunological effector function of specific rabbit polyclonal anti-SesC IgGs, we performed an *in vitro* opsonophagocytosis assay.

7.2 Materials and Methods

7.2.1 Neutralizing antibody assays

In this study, a neutralizing antibody assay was done to check whether the polyclonal antibody against SesC has a neutralizing effect. To this purpose, we compared the rate of growing of *S. epidermidis* with or without this antibody.

This experiment was done on two *S. epidermidis* strains with different mechanism of biofilm (1457, PIA dependent and eDNA, and st8, a clinical isolate with proteinaceous biofilm). Briefly, the overnight cultures were diluted to the OD_{600}=0.001 in BHI medium. At several time points, including 0', 30', 1 h, 2 h, …, 10 h, the OD in the presence or absence of anti-SesC-IgGs (30 ug/) was checked.

7.2.2 Opsonophagocytosis assay

To prepare the bacteria for the evaluation of susceptibility to opsonic killing, cells from an overnight culture of *S. epidermidis* 10b in BHI were pelleted for 5 min at 12,000 *g* at 4°C, washed twice with PBS, and subsequently diluted to an OD_{600} of 1. For evaluation of opsonophagocytosis of planktonic cells, 10 µlof bacterial suspension was used, and for evaluation of opsonophagocytosis of biofilm cells, 5-mm catheter (Arrow International) fragments were added to the bacterial suspension, incubated at 37°C for 2 h, and subsequently washed with 1 of PBS. The opsonophagocytosis assay was performed with fresh blood obtained from human healthy volunteers as previously described (Smith et al. 2012), with some modifications. Briefly, fresh whole blood from two volunteers was collected in vacuum blood collection tubes containing the anticoagulant heparin and then aliquoted into 1.5- microcentrifuge tubes (0.5 /tube). Catheter fragments pre-incubated with

S. epidermidis strain 10b or planktonic bacteria (10 µlof bacterial suspension with an OD_{600} of 1) after 1 h pre-incubation at 4°C with pre-immune or anti-SesC-IgGs (30 ug/) or the same volume of PBS, or nothing, were added to the 1.5-ml microcentrifuge tubes containing 0.5 ml of fresh blood. Tubes were incubated at room temperature with gentle rocking and, after 30 min, the samples containing planktonic bacteria were serially diluted and plated onto TSA plates to determine the number of surviving CFU. For samples containing catheter fragments, catheter fragments were first removed and gently washed with 1 ml of saline and then processed for quantification of the number of surviving cells on the catheter fragments as explained in chapter VI. In order to determine the input CFU in samples containing planktonic or sessile bacteria attached to the catheter fragments, a portion of untreated planktonic samples was serially diluted and plated onto TSA plates, or the number of bacteria attached to the untreated catheter fragments was quantified as explained above. All samples were assayed in triplicate, and all experiments were repeated at least twice (Shahrooei et al. 2012).

7.3 Results

7.3.1 Polyclonal antibody against SesC does not have a neutralizing effect

In this experiment, the rate of growing of bacteria in different time points in the presence or absence of antibody was checked. The results showed no changes in the growing of these bacteria in presence of antibody (Figure 7.1). This suggests that this antibody has no neutralizing effect.

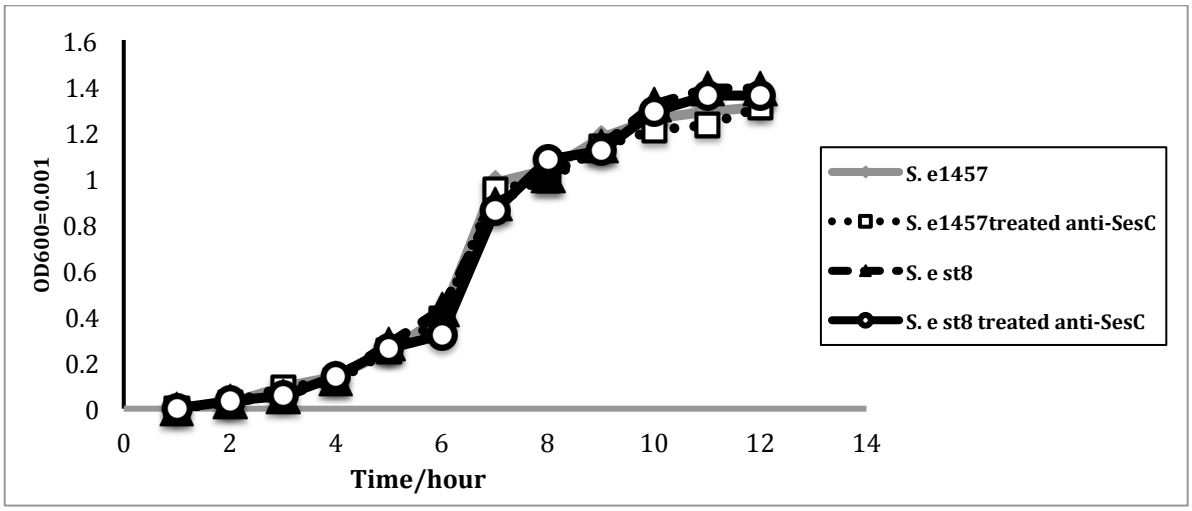

Figure 7.1. Neutralizing effect of anti-SesC-IgGs on *S. epidermidis* growth. The growth rates of planktonic bacteria (starting OD_{600}=0.001) were checked in presence of anti-SesC-IgGs. These results are the average of three different biological repeats.

7.3.2 αSesC-IgG antibodies has a opsonic effect

Incubation of bacteria in the planktonic or sessile state with whole blood for 30 min led to a significant reduction (>1 $\log_{10}$) in PBS-treated versus untreated bacteria (P<0.001) (Figure 7.2). Pre-incubation of bacteria in both planktonic and sessile forms with αSesC-IgGs significantly enhanced the opsonophagocytic killing of bacteria (P<0.001, one-way ANOVA) compared to bacteria treated with pre-immune IgGs or PBS (Figure 7.2). There was no difference between bacteria treated with pre-immune IgGs and PBS. αSesC-IgGs showed the same level of enhancement of opsonophagocytic killing of bacteria for both planktonic and sessile bacteria (Shahrooei et al. 2012).

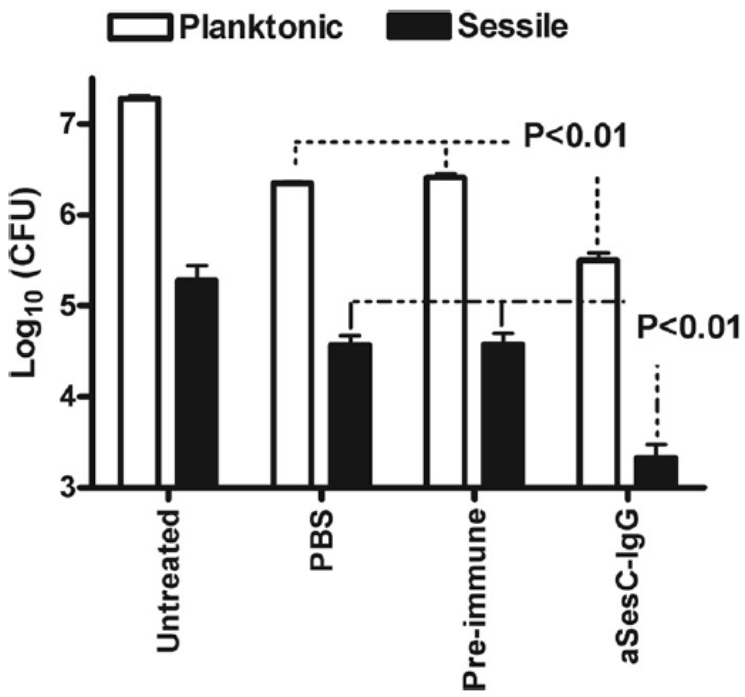

Figure 7.2. Effect of pre-incubation of *S. epidermidis* strain 10b with αSesC-IgGs on phagocytosis of planktonic and sessile bacteria by human neutrophils. Bacteria grown in planktonic form or attached to catheter fragments were tested for their ability to survive in human blood after pre-incubation with pre-immune IgGs or αSesC-IgGs. Surviving bacteria were measured by viable counting. The data are presented as the mean log10 of surviving CFU. The data for each group are the average of six measurements in two independent experiments. The error bars represent the standard deviation of the measurements.

7.4 Discussion and Conclusion

The primary functions of antibodies are, to a lesser extent, neutralization and more importantly, opsonization. The difference between a neutralizing antibody and a binding antibody is that neutralizing antibodies neutralize the biological impact of the antigen, while binding antibodies mark antigens to the white blood cell. Since the αSesC-IgGs don't have any effect on the growth of the bacteria, a neutralizing effect seems less likely.

The effect of αSesC-IgGs on *S. epidermidis* biofilms *in vitro*, in the absence of immune system components, suggested a neutralizing effect of αSesC-IgGs or blocking of the function of SesC. However, *in vivo*, in addition to neutralization, antibodies can opsonize their ligand, thus facilitating its uptake and destruction by natural killer cells, activating complement, and enhancing phagocytosis. The effect of αSesC-IgG antibodies on opsonophagocytosis of planktonic cells *in vitro* indicates their potential opsonic activity, whereas the reduction of the number of sessile bacteria on catheters in this experiment can be due to both neutralization and the opsonic activity of αSesC-IgGs. It is also possible that binding by αSesC-IgG to SesC on the surface of sessile bacteria triggers their detachment from catheter fragments and subsequent phagocytosis by neutrophils (Shahrooei et al., 2012).

CHAPTER VIII: Production of monoclonal antibodies against SesC

8.1 Introduction

To evaluate the potential use of SesC as a vaccine target, we set out to make a monoclonal antibody against this protein. This monoclonal antibody may help us to find the best epitope of SesC for vaccine development. The most common epitope mapping approaches are the generation of consecutive, overlapping synthetic peptides (usually <15 aa) which cover the complete primary sequence of the protein antigen or using a bacterial surface display of antigen protein fragments. Both are usually followed by screening for antibody binding by ELISA and western blotting (Mirshahidi et al., 2009; Hudson, Uhlen, & Rockberg, 2012). As a future perspective, it would be interesting to use both methodologies to identify linear and conformational epitopes of this target.

8.2 Materials and Methods

To obtain monoclonal antibodies, we used the Hybridoma technology. A hybridoma is a cell that can be generated by fusing a spleen cell from an immunized animal with a Myeloma cell and that is capable of secreting permanently in culture, a monoclonal antibody specific to one epitope of the antigen. This technique follows the steps summarised below (Figure 8.1).

8.2.1 Purification of recombinant protein

Construction and purification of histidine-tagged fusion SesC proteins was done as explained (Chapter V); briefly, the recombinant extracellular domain of the SesC protein was expressed with an hexahistidine tag at its C terminus using the expression vector pET11c (Novagen, Madison, WI). Forward (ACGTGCTAGCGCAGATTCAGAAAGTACATC) and reverse (ATGC*GGATCC*TAGTGATGGTGATGGTGATGATCAGCTGTAGCTGTTCC) primers with incorporated flanking *Nhe*I and *Bam*HI restriction sites (underlined) and a sequence coding for a C-terminal His6 tag (in italic and bold) were used. The *sesC* amplicons were cloned in pET11c and the resulting plasmids (pET11c-sesC) were transformed into *E. coli* BL21 (DE3) (Hermans et al. 2006). The resulting plasmids were used for recombinant protein production.

Recombinant protein expression was achieved as previously described (Hermans et al. 2006). Briefly, after transformation, *E. coli* BL21 (DE3) was grown with shaking (250 rpm) at 37ºC in Luria-Bertani (LB) broth with 100 µg/ml ampicillin to an OD_{600} of 0.6-1.0. Expression was induced by addition of 1 mM IPTG for 2 h. After cooling on ice, cells were harvested by centrifugation (4000 rpm, 10 min at 4ºC) and resuspended in 5 ml imidazole

buffer (20 mM phosphate, 0.5 M NaCl, 10 mM imidazole) and frozen at -20ºC. Cells were first thawed on ice and then sonicated 3 times for 30 s. After centrifugation (30 min, 15000 rpm at 4ºC) the supernatant was used for Ni+ affinity chromatography purification of the recombinant proteins with the HisTrap™ Kit (Amersham Pharmacia, Uppsala, Sweden). The columns were washed with 40 mM imidazole buffer and proteins were eluted with 300 mM imidazole buffer. The purified recombinant protein was dialyzed against 10 mM HEPES buffer, pH 7.5, freeze dried, and stored at -20 °C.

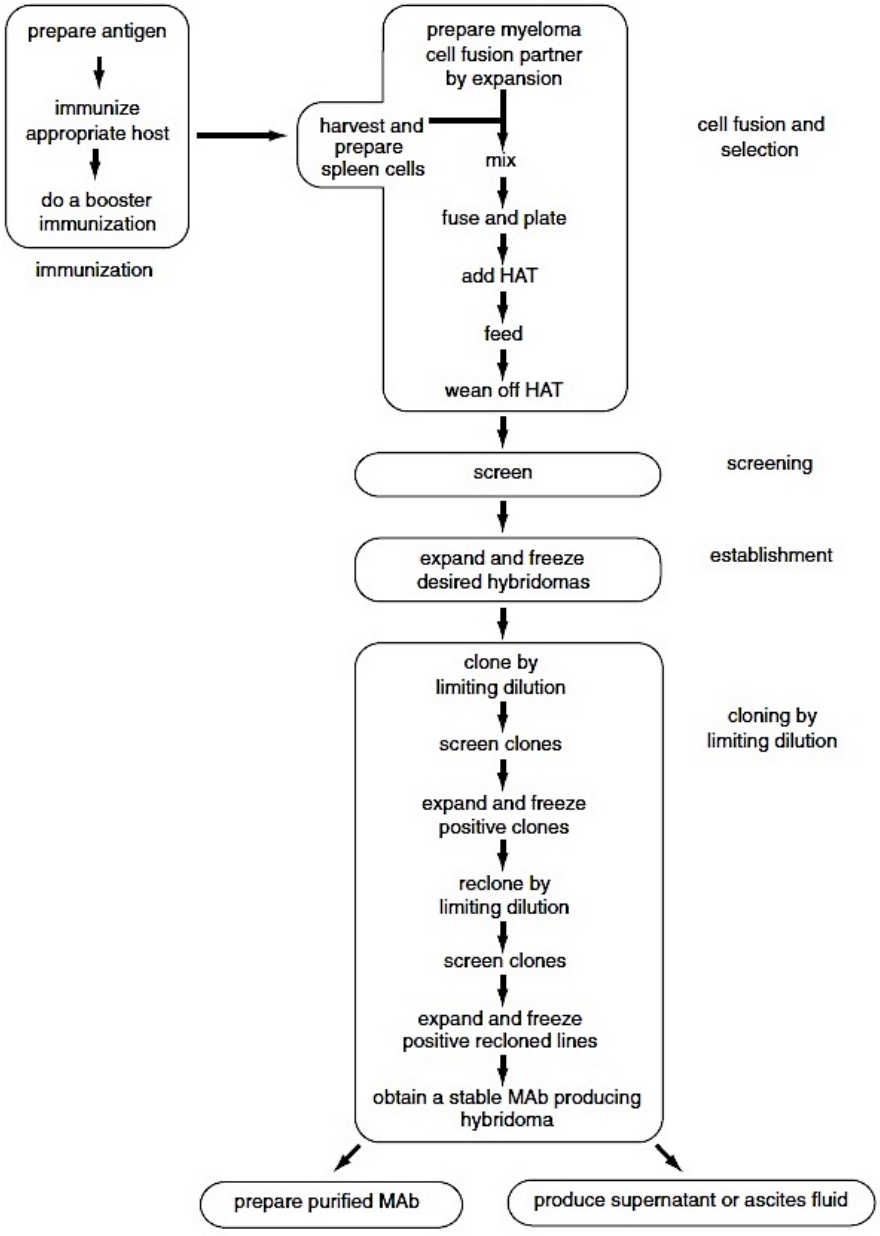

Figure 8.1. Stages of monoclonal antibody production, (van Ewijk 1985) .

8.2.2 Immunization

Immunization is one of the most important steps. In this step, 50 µg recombinant protein (r-SesC) were mixed well with complete Freund's adjuvant (CFA) in equal volume and injected intraperitoneally into each mouse (5 weeks Swiss mouse strain). Then after 10 days post injection, the mice were boosted with approximately the same dose of antigen mixed with incomplete Freund's adjuvant (IFA) (van Ewijk 1985).

Seven days after the boost immunization, blood was taken and tested for a positive immune response against the respective antigen, by ELISA. Four weeks later, the mice were boosted by third intraperitoneal injections of 50 µg antigen in saline four days before cell fusion.

8.2.3 Hybridoma production

One week before fusion, we expanded the SP2/0-Ag14 myeloma cell line (the fusion partner cell line). On the day of fusion, the boosted animals were sacrificed and the spleen was aseptically harvested and put into a petri dish containing RPMI-1640 medium (Sigma-Aldrich). Under a laminar flow hood, as many spleens cells as possible were removed from the capsule. Cells were mixed very gently and transferred to 15 ml tube containing medium. The myeloma and spleen cells (Figure 8.2) were counted and adjusted to 1:10 ratio myeloma/spleen. The cells were centrifuged and washed with 10 ml of the medium without FBS and subsequently the two cell lines were mixed into one 50ml centrifuge tube and centrifuged 5 minutes at 300 *g*. The pellet was re-suspended with 1.2 ml of PEG very gently in a water bath. Then, first 1ml RPMI without FBS was added to the tube very gently over 1 min. In succession, this step repeated with 2 ml, 4 ml, and 8 ml of serum free medium and kept in a water bath for 15 min. Afterwards, the cells were centrifuged for 5 min at 300 *g*, and very gently resuspended in 10 ml of RPMI containing 1% Ultroser G (Pall Ref. 15950-017), 10% FBS (Genycell Ref. GCS0101-500) and antibiotics (100 U/mL Penicillin and 100 µg/mL Streptomycin), followed by adding 190 ml of the same warm medium and was aliquoted 1 ml of the suspension into each well (8 x 24-well plates).

24 h after fusion, 1 ml of the medium with HAT (Hypoxathine, Aminopterin, Thymidine) (Gibco Ref. 21060-017) was added and the plates were left in the incubator till colonies appeared after approximately 14 days (http://www.euromabnet.com/protocols/).

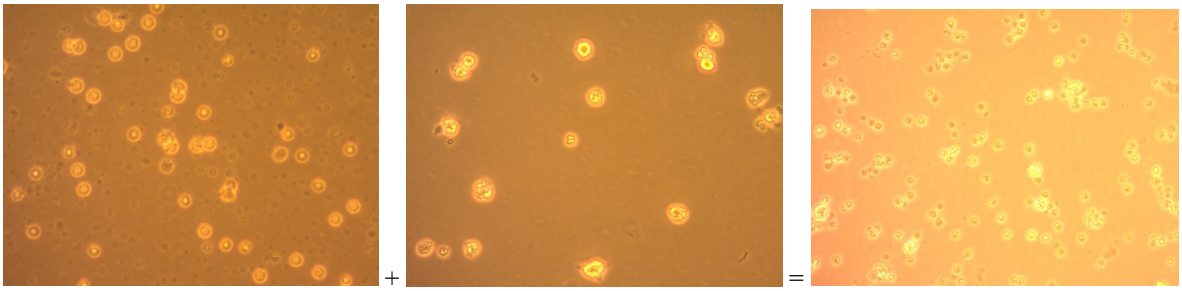

Figure 8.2. Live microscopy images of splenocytes, myeloma cells and hybridoma cells (40x magnification).

8.2.4 Screening and Cloning

Most wells may not contain the desired antibody or may contain nonproducing hybridomas. Screening is done to determine which wells contain hybridomas that secrete the desired specific antibody.

To this purpose, first we checked and estimated the number of growing positive hybridomas using a microscope and performed ELISA. When cells in the 24-well plates were 25% to 50% confluent, they were frozen and used for cloning by limiting dilution.

We followed the cloning procedure to ensure that the desired hybridoma cell line produced is obtained from a single fused cell. The method that we used for cloning was that of limiting dilutions. This was done for all candidate cell lines. The candidates were re-cloned and ELISA was performed by taking 100 µl of the medium of each clone. This step was repeated and we got the best hybridomas cell line.

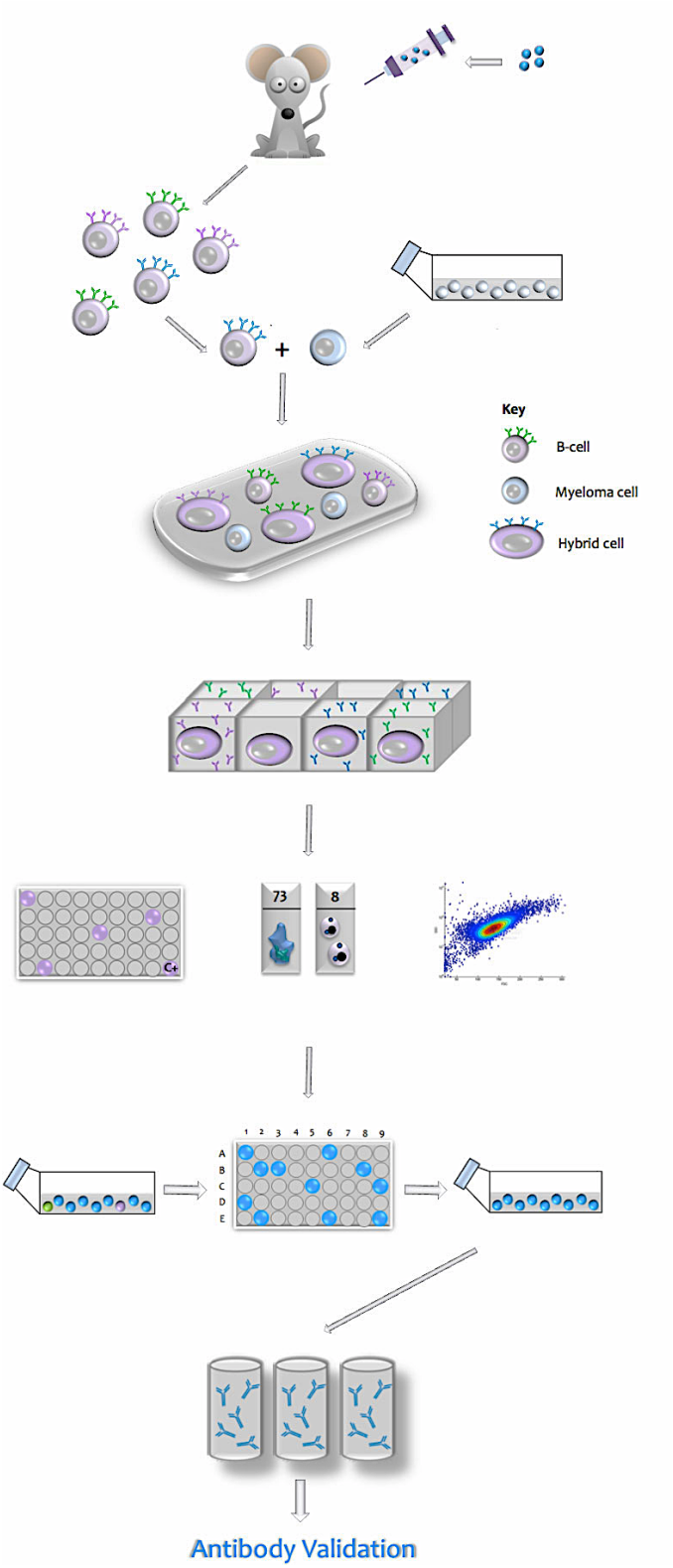

Figure 8.3. Schematic representation of the monoclonal antibodies production protocol used in this study, which contains all the steps such as immunization, hybridoma production, screening, cloning, expanding and freezing down hybridomas, and antibody validation required for the production of a high quality monoclonal antibodies (https://www.euromabnet.com/protocols/).

8.2.5 Antibody purification

The antibody was purified using Protein G mag sepharose (GE Healthcare). First the supernatant of selected cells was mixed with 100 µl of Protein G mag sepharose in an Eppendorf tube, then incubated whilst rotating for 1 h. Subsequently, the Eppendorf tubes were placed in a magnetic rack, the unbound fraction was discarded and the bead-bound antibodies were extensively washed with 1x PBS. IgGs were eluted subsequently with 0.1 M glycine/HCl buffer at pH 2.7, and the eluted part was immediately neutralized with 1 M Tris buffer (pH 8.5). The total IgGs from the serum of the hyper-immunized mice (from the blood of mice that served as splenocyte donors and contained hyperimmune serum) was also purified as a control, and all purified IgG was stored at -20°C. Then 10 µlfrom each purified IgG were loaded on the SDS gel and stained. ELISA and Western blot were also performed with this IgG, as primary antibody, and HRP-mouse secondary antibody was always used to detect primary antibody attached to SesC protein. Then by pipetting 100 µl of TMB Reagent after sufficient color development, 100 µl of Stop Buffer added to each well and the plate were read in the absorbance of 450 nm.

8.3 Results

To generate MAs towards surface protein SesC, five Swiss mice were immunized with r-SesC and after 14 days the ELISA results confirmed the successful immunization in all five mice.

Consecutive fusions were performed first for spleens of 2 mice and the resulting hybridoma colonies were screened for expression of antibodies against SesC, but no positive hybridoma colonies were observed. In the second attempt, 50 colonies out of 70 colonies were positive and the antibodies produced by the hybridoma colonies (Figure 8.4) were tested by Western blot (Figure 8.5). The best colonies were expanded and frozen. These MAs will be further characterized.

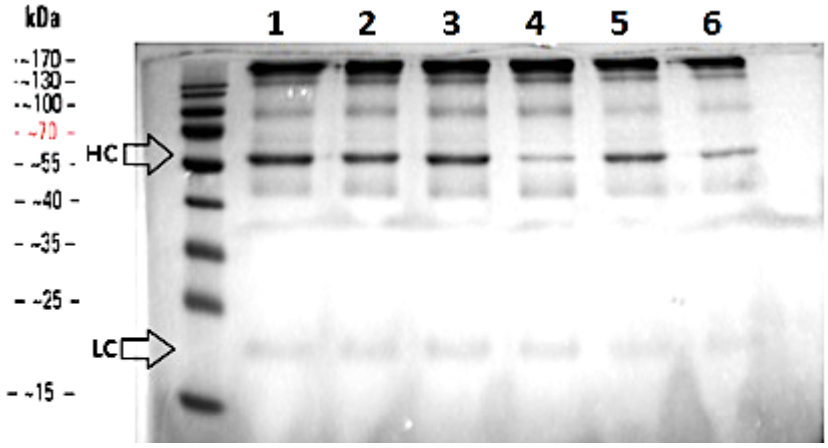

Figure 8.4. The Coomassie stained gel contains IgG purified by G mag sepharose. In order to evaluate expression levels of antibody, we loaded 10 µl of the purified IgG on the SDS gel. Numbers from 1 to 6 shows different IgG achieved from different cell lines. According to the known structure of IgG, the heavy chain (HC) and light chain (LC), are shown by arrows.

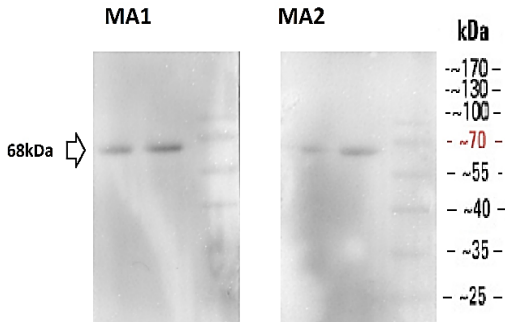

Figure 8.5. Western blot analysis performed for rSesC by the MAs. The 68 kDa band contains SesC protein, and is detected by the IgG purified from two different hybridoma cell lines.

8.4 Discussion

To recognize the best epitope of SesC, we made monoclonal antibodies against this protein. Polyclonal antibodies are the antibodies of choice when we need to recognize multiple epitopes of the antigen while the monoclonal antibodies help to recognize one single epitope. In addition, every batch of monoclonal antibodies has no or very low variability.

As explained in this chapter, we followed a standard monoclonal antibody protocol to find candidates for the epitope mapping of SesC. We obtained several hybridoma cell lines with SesC positive ELISA's and Western blots and we got one specific band for SesC at 68 kDa. To confirm the specificity of antibodies further assays such as mass spectrometry and FACS should be done.

All the experiments that have been done with polyclonal antibody should be repeated for these monoclonal antibodies to see if the biofilm and the rate of infection in the infected mice are significantly reduced.

General Discussion

S. epidermidis is a skin colonizer that apparently evolved as a non-pathogenic species. However, these last decades, nosocomial infections by these bacteria got more and more frequent, so they obtained much attention.

Different factors in these bacteria help them to easily attach to the surface and also attaching to each other and make strong biofilms. It was shown that surface proteins have important roles in helping these bacteria to make biofilms (Foster et al. 2014).

It was reported that polyclonal antibodies against surface protein SesC can significantly reduce the biofilm of *S. epidermidis* (Shahrooei et al. 2009). In the current study, we tried to explain how this protein could be involved in biofilm formation.

To understand this, we first tried to find a *sesC* natural mutant in *S. epidermidis*, to check how absence of SesC, can affect the biofilm of these bacteria. To this purpose, 175 *S. epidermidis* isolates were collected from the patients in UZ Leuven hospital and identification of all was confirmed by MALDI-TOF/TOF mass spectrometry and all isolates were checked for the presence of *sesC* by a colony PCR assay. The results showed that *sesC* was present in all isolates. Then we checked another 263 CoNS and *S. aureus* for *sesC*, but the results showed that *sesC* is specific to *S. epidermidis*.

Knockout of a gene can help to learn about the function of a protein. We attempted to knock out the *sesC* gene in *S. epidermidis*, but in spite of sustained efforts, we were unsuccessful. We then used antisense RNA, and that also didn't work. These results suggest that *sesC* is a conserved gene in *S. epidermidis*, and maybe it is also an essential gene.

Subsequently, we used an alternative strategy, which was expressing SesC in *S. aureus* and we examined the changes in the biofilm formation. Since the mechanisms of biofilm formation in *S. epidermidis* and *S. aureus* are similar, we transformed *S. aureus* strains with different mechanisms of biofilm formation (proteinaceous or PIA-dependent), and examined the effects.

It was shown before that different compounds and growing conditions affect the expression of genes and these in turn will affect the quality of biofilm and can even change the

phenotype of biofilm. Environmental factors such as sugar, salt, pH and other nutrients have been demonstrated to impact on biofilm formation (Pan et al. 2010). The presence of glucose or NaCl can influence biofilm production. NaCl represses proteinaceous biofilm by activation of the accessory gene regulator (*agr*) system, and stimulates PIA-dependent biofilm via activation of SigB that inhibits transcription of *icaR*, a regulator of *icaADBC* operon expression (McCarthy et al. 2015). Glucose induces biofilm by stimulation of *sarA* expression and interestingly by acidification of the culture media (Rode et al. 2007; Conen et al. 2008; O'Gara 2007).

Expression of SesC in *S. aureus* BH1CC that normally expresses a protein-dependent biofilm in BHI medium containing glucose or NaCl, didn't show any differences in comparison to the wild-type strain or a strain transformed with a mock plasmid. However, transformants of *S. aureus* 8325-4 with *sesC* could not make biofilm in the presence of NaCl.

Treating the biofilms of these transformant strains in the presence of glucose with sodium metaperiodate or proteinase K showed that expression of SesC caused switching of the PIA-biofilm to a protein-dependent biofilm. This phenomenon also happened when *S. aureus* was overexpressing the surface protein SasG (Corrigan et al. 2007). These data show that some surface proteins have dominant roles in biofilm formation, even when there is expression of *ica* genes. In another series of experiments, another *S. epidermidis* surface protein, *sesK*, was transformed into this *S. aureus* strain as a control. Antibodies against SesK did not affect the biofilm in *S. epidermidis*. Transformation of *S. aureus* 8325-4 with *sesK* didn't affect the biofilm formation of this strain.

Our dot-blot results for the expression of PIA in transformant strains with *sesC* compared to wild type showed that SesC didn't have any effect on the expression of PIA. However, since in the presence of NaCl, we found a big reduction of biofilm amount, there should be a defect in the sorting of PIA to the surface in the presence of SesC. That may be due to an effect on IcaC or IcaB, since they have important roles in transferring PIA to the surface.

To confirm this, *S. aureus* 8325-4::*ica*, which is a non-biofilm forming strain, was transformed with *sesC* and we checked whether there were any changes in biofilm formation. SesC induced this non-biofilm forming strain to form a protein-dependent biofilm.

To further answer the question if SesC can directly affect biofilm formation and if these changes are due to the presence of SesC on the surface, *S. aureus* strain 8325-4 with *srtA*

mutation was transformed with *sesC*, and interestingly SesC switched this biofilm forming strain to a non-biofilm forming strain. To be sure that in this strain SesC could not sort to the surface, we separated the soluble proteins from insoluble proteins. Since the membrane and cell wall anchored surface protein are in the insoluble fraction, our results confirmed that SesC is expressed but is mostly present in the soluble part and not in insoluble fraction. The switch of *S. aureus* 8325-4 *srtA::tet* (pCNsesC) to a non-biofilm forming strain suggests that the presence of SesC in the cytoplasm leads to PIA sorting defects. Complementation of the non-biofilm-forming *S. aureus* 8325-4 *srtA::tet* (pCNsesC) with *srtA* by means of transformation with pSRsrtA5 turned it to a proteinaceous biofilm-forming strain. These data confirm that transformation with *sesC* is sufficient to switch the mechanism of biofilm formation to a proteinaceous type biofilm on condition that SesC is sorted to its place on the surface.

It was shown that some MRSA strains naturally show different characteristics to make biofilm in medium containing glucose or NaCl. This suggests that depending on environmental conditions, different expression of proteins can lead the strains to form different types of biofilm. For example, strain *S. aureus* S115 generates PIA but is a non-biofilm-forming strain. This may be because of the existence of a defect in the export of PIA by IcaC or IcaB (Vergara-Irigaray et al. 2009). Similarly to the effect of FnAB on biofilm phenotype in *S. aureus* strain S132 and BH1CC, there is the possibility that the extracellular location of SesC changes the architecture of the cell wall to the extent that PIA can no longer link the cells. This hypothesis is consistent with the observed effect of biofilm dispersal agents on *sesC*- and *ica*-positive strains (Vergara-Irigaray et al. 2009).

We also used a polyclonal antibody against SesC on the biofilms of strains transformed with *sesC* and compared this effect with the impact on the strain transformed with *sesK* and wild-type biofilms. The results showed a significant reduction of biofilm but only in the strain in which SesC was expressed.

It was also shown that active immunization with the recombinant SesC significantly decreases the *S. epidermidis* biofilm formation in a subcutaneously catheterized rat model (Shahrooei et al. 2012).

In this study, we developed a jugular vein catheterized mouse model and we infected the mice with an *S. epidermidis* strain and checked the influence of polyclonal antibody against SesC. We observed a significant reduction of infection in the organs and catheter compared to the untreated mice. Following this, we infected the mice with *S. aureus* transformed with

SesC, SesK, and mock plasmid and treated them with this antibody. These results again proved the important role SesC can play in staphylococcal infection.
To understand the effector function of this antibody, we performed different assays, such as neutralization and opsonophagocytosis assays, and the results suggest that this antibody has an opsonophagocytic killing.
Although we have to be cautious in extrapolating conclusions based on data obtained in *S. aureus* to *S. epidermidis*, we suggest that SesC is a virulence factor associated with the early stages in *S. epidermidis* biofilm formation, such as adhesion and colonization. This way SesC may favor chronic, persistent infections on indwelling biomaterials. The biofilm formation abilities of *S. epidermidis* may be due in part to the presence of SesC and similar factors (Khodaparast et al. 2016b).

Future perspective

This study showed that SesC can be considered as a potential target for immunization against *S. epidermidis* infection. Further work should be directed to finding the best epitopes of SesC. To this end, more work has to be done, such as:

- Constructing monoclonal antibodies against SesC that can be used for *in vivo* studies and that can reduce the rate of infection in the infected mice as well as the polyclonal antibody and using these monoclonal antibodies to find the most immunogenic epitope.
- Investigating the mechanism of action of the antibodies; for example blocking of the targeted molecule functions, or modulation of signaling pathways.

Since biofilm formation in *S. epidermidis* is a complicated process with many different factors involved, different targets for immunization may be present. The best candidate epitopes from the most important factors involved may be combined and used as an antigen to vaccinate against these bacteria.

Considering our unsuccessful efforts to knock out SesC in these bacteria, we believe that *sesC* is an essential gene. To understand the role of SesC, we may have to study its role in a broader way instead of just focusing on the function in the biofilm formation.

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Curriculum Vitae

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Education

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Joined to the Switch Laboratory **2015-2017**

VIB-KU Leuven Center for Brain & Disease Research-Department of Cellular and Molecular Medicine

KU Leuven, Belgium Faculty Of Medicine **2012-2017**

Department Of Clinical Microbiology & Immunology

Pre-doctoral program in Biomedical Science **2010-2012**

KU Leuven, Belgium, Faculty of Medicine, Department of Clinical Microbiology & Immunology

M.Sc in Biological science **2006-2008**

Azad University of Kazeroon, Iran, Department of Physiology

B.Sc. in Biological science- Zoology **2002-2006**

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Awards

- Travel Grant, 50th ASN kidney week, 15-21 Nov. 2, Chicago, US (2016 November)
- Poster winner, Fifth PHD symposium VIB Neuroscience-NERF (2016 September)
- Top Speaker, Fifth PHD symposium VIB Neuroscience-NERF (2016 September)
- Travel grants of 24th ECCMID 2014, the European Society of Clinical, Microbiology and Infectious Diseases (ESCMID) (2013-2014)
- Top student: Awarded first rank among M.Sc graduating in the Biology in university (2006-2008)
- Top student: Awarded second rank among 900 participants for entrance without exam in IRAN for M.Sc degree between all Azad University (2006-2008)
- Top student: Selected as one of the elite student in 2008 in Fars province, Iran (2006-2008)
- Top student: Awarded first (2005-2006)

PUBLICATIONS

- Elmonem M., Khalil R., Khodaparast L, **Khodaparast L.,** Van den Heuvel L. P., Levtchenko E. (2017). **Cystinosis (ctns) zebrafish mutant shows pronephric glomerular and tubular dysfunction.** *Scientific Report,* 7:42583. doi:10.1038/srep42583 **(IF: 5.22).**

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- Mokhtari M., Shariati M., **Khodaparast L.; The effect of hydro-alcoholic extract *Mentha pulegium* leaves on liver function test in adult male rats Iranian Journal of Sabzevar University of Medical Sciences**, 2008, 15(2).

Conferences and Lecture Presentations

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- **Khodaparast, L**., Khodaparast, L., Gallardo, R. Schymkowitz, J., Rousseau, F. Van Eldere. Targeted protein aggregation as antimicrobial agents strategy in vitro. VIB Neuroscience-NERF PhD student symposium, Oral presentation, 30th September 2016, IB11 - O&N 4, Leuven, Belgium.

- Khodaparast, L., **Khodaparast, L**., Gallardo, R. Schymkowitz, J., Rousseau, F. Van Eldere. Targeted protein aggregation as antimicrobial agents strategy in vivo. VIB Neuroscience-NERF PhD student symposium, Poster presentation, 29th September 2016, IMEC-NERF, Leuven, Belgium.

- Khodaparast, L., **Khodaparast, L.,** Shahrooei, M., Gallardo, R. Schymkowitz, J., Rousseau, F. Van Eldere. (2015). Oral presentation, **Induction of protein aggregation as a novel antibiotic mechanism**. ISM Congress16. 25th August 2015.

- **Khodaparast, L**., Khodaparast, L., Van Eldere J. (2015). Oral presentation, **LPXTG surface protein SesC as a vaccine target against *Staphylococcus epidermidids* infection**. ISM Congress16. 26th August.

- Khodaparast, L., **Khodaparast, L.,** (2015). Evaluation of the activity of new antimicrobial agent, workshop organizer and teacher, Institute of Medicinal Plants Ground floor, August 25th.

- **Khodaparast, L.,** Khodaparast, L., **(**2015). **How to study biofilms in vitro and in vivo,** workshop organizer and teacher, Institute of Medicinal Plants Ground floor, August 26th.

- **Khodaparast, L.,** Khodaparast, L., Shahrooei, M., Van Eldere, J. (2014). **Rapid identification of *Staphylococcus epidermidis* by colony PCR with *S. epidermidis* surface protein SesC as a marker**. ECCMID. Barcelona, 10-13 may 2014.

- **Khodaparast, L.,** Khodaparast, L., Shahrooei, M., Van Eldere, J. (2014**). Evidence for the involvement of *Staphylococcus epidermidis* LPXTG surface protein SesC in biofilm formation and catheter-related infections.** ECCMID. Barcelona, 10-13 may 2014.

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- **Khodaparast, L.,** Khodaparast, L., Shahrooei, M., Van Eldere, J. (2013). **Evidence For The Involvement Of *Staphylococcus epidermidis* LPXTG Surface Protein SesC In Biofilm Formation.** ICAAC. Denver, 10-13 September 2013.

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ACADEMIC PROFESSIONAL EXPERIENCE

- 25thAugust 2015 Workshop organizer and presenter, the 16th international and Iranian Congress of Microbiology, Evaluating of the new antimicrobial agents, http://16.ismcongress.ir/En_Default.aspx?PN=82
- 26thAugust 2015 Workshop organizer and presenter, The 16th international and Iranian Congress of Microbiology, How to study biofilms in vitro and in vivo, http://16.ismcongress.ir/En_Default.aspx?PN=82

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THE POSSIBLE ROLE OF THE STAPHYLOCOCCUSE EPIDERMIDIS LPXTG SURFACE PROTEIN SESC IN BIOFILM FORMATION AND ITS USE AS A POTENTIAL VACCINE TARGET

FACULTY OF MEDICINE
DEPARTMENT OF CLINICAL
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MAY 2017